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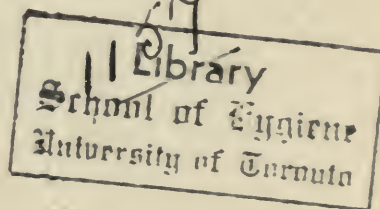
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ANTIBODIES IN THE FETUS

HAL W. SHERMAN

From the John McCormick Institute for Infectious Diseases, Chicago

The scope of this paper is confined to a study of the natural antibodies of swine embryos. Included are lysins, complement and bacterial opsonins.

TECHNIC

Hemolytic tubes of 4 c c capacity were used; 0.2 c c of a 2½% erythrocytes suspension, the required amount of embryonic serum, amniotic, or allantoic fluids, and either 1 unit of complement or lysin were placed in the test tubes and sufficient isotonic NaCl solution added to make 1 c c. After shaking, the tubes were placed at 37 C. for 1 hour, shaken again and reincubated for 1 hour. The results were then read and the tubes placed in the cold over night when any change from previous reading was noted.

Guinea-pig complement was used for all except guinea-pig erythrocytes. For these, complement was obtained by absorption in the cold of fresh sheep or rabbit serum with a heavy suspension of guinea-pig erythrocytes. The salt solution was always tested for isotonicity as were also the amniotic and allantoic fluids.

The results are expressed as follows: (+ + +) means complete, (+ +) partial, (+) slight, (0) no hemolysis. In all the tables the embryos are arranged in order such that the youngest is at the top and a gradual transition to the oldest at the bottom.

Mendel and Mitchell¹ compiled the following table by which the age of swine embryos may be judged by their length from the end of the snout to the base of the tail with the head in flexion.

Average length in mm.	Age in days
25	32
50	44
75	54
100	62
125	68
175	80
200	88
230	96
280	110
300	112

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¹ Am. Jour. Physiol., 20, p. 81.

The material for these experiments was obtained from the Chicago stockyards through the courtesy of Dr. Enos Day and Armour & Co. In all cases mature swine serum was secured at the same time as the fetal serums for a control. The allantoic and amniotic fluids were collected with special care that they were not contaminated with maternal blood. The fetal blood was collected from the umbilical cord and from the veins of the neck also when necessary. The blood from all the fetuses of a litter was mixed together and the average length of the fetuses taken.

TABLE 1
FRESH SERUM OF SWINE EMBRYOS

Number of Embryo	Length of Embryo in Cm.	Complement Titers Action of Serum + 1 Unit of Amboceptor on Erythrocytes of				Lysin Titers Action of Serum + 1 Unit of Complement on Erythrocytes of			
		Sheep	Goat	Guinea-pig	Rabbit	Sheep	Goat	Guinea-pig	Rabbit
25	8			0				0	0
30	10					0	0	0	0
2	11	0	0	0	0	+++	+++	0	
28	14					0	+++	0	0
10	15	0	0	+++	0	++	0	+++	++
27	17					0	0	+++	0
16	17.5	0	0	+++	0	+		+++	++
20	18	0	0	+++	++	0	+++	0	0
24	19	0	0	0	0	0	+++	0	0
6	20	0	0	0	0				
9	20					0	0	+++	0
18	21.5	0	0	+++	0	+++	0	+++	+++
22	22	0	+	+++	+++	0	+++	0	0
26	22.5	0	0	++	++	0	+++	0	0
20	25	0	0	+++	0	+	0	+++ (0.05)	0
12	26.5	0	0	+++	0	+	0	+++ (0.05)	+++
8	27					0	0	+++	0
17	27	0	0	+++	0	+	0	+++	+++
10	28	0	0	+++	0	0	0	+++	0
1	30					+	+	0	
14	30	0	0	+++	0	0	0	+++ (0.05)	+++
19	30	0	+++	+++	0	0	0	+++	+++
21	30	0	0	+++	0	0	0	+++ (0.1)	+++
11	31.5	+++	+++	+++ (0.1)	0	++	0	+++ (0.05)	++
7	34					0	0	+++	0
15	35	0	+++	+++	0	0	0	+++ (0.05)	0

Unless otherwise stated the amount of embryo serum was 0.5, 0.4 or 0.3 c.c.; the figures in parentheses denote in c.c. any other amount used.

Table 1 gives the complement titers of the blood serums of the swine embryos. No complement could be demonstrated in the 2 youngest embryos, which were approximately 8-9 weeks old. Complement for guinea-pig erythrocytes was present almost constantly in all serums after the 11th week; for sheep erythrocytes only in 1 of the

oldest; for goat erythrocytes in 3 of the oldest; for rabbit erythrocytes in 3 out of the 5 serums to which lysin was added. Thus we observe an increase of the different kinds of complement at the period when the fetus has reached its maximum intra-uterine development. However, the complement that is present from early embryonic life does not increase materially in titer at this later period, and averages only 10% of that of the mature swine serum (Table 2).

Table 1 also gives the lysin titer of the same embryo serums. The picture presented here varies somewhat from that of the complement. Previous to the 13th week of gestation only 4 serums contained complement, while 13 contained lysin. The lysins for sheep and goat erythrocytes were not found with any degree of regularity, and this was true of the complement also. The lysin titers averaged less than 4% of those of the mature swine serums.

TABLE 2
ACTION OF FRESH SERUM OF MATURE SWINE ON VARIOUS ERYTHROCYTES

Swine Number	Lysin Titers of Swine Serums for Erythrocytes of				Complement Titers of Swine Serums for Erythrocytes of			
	Sheep	Goat	Guinea-pig	Rabbit	Sheep	Goat	Guinea-pig	Rabbit
1	0.02	0.02	0.02		0.001	0.005	0.02	
2	0.05	0.03	0.03		0.005	0.005	0.005	
3	0.01	0.01	0.03		0.005	0.003	0.005	
4	0.03	0.03	0.05		0.010	0.005	0.003	
5	0.03	0.03	0.03	0.1	0.005	0.005	0.003	0.02
6	0.03	0.03	0.03	0.1	0.01	0.01	0.003	0.02

The figures given refer to the amount in cc of fresh serum of mature swine required to completely hemolyze 0.2 cc of a 2½% suspension of the corresponding erythrocyte. One unit of complement was added to each tube titrated for lysin. In the titration for complement there was always present an excess of native lysin, making it unnecessary to add more.

AMNIOTIC FLUIDS

The amniotic fluids were taken from the same uterus as the correspondingly numbered embryos.

Complement for sheep, goat, guinea-pig, and rabbit erythrocytes was tested for in 26 fluids; it was demonstrated in but two (9 and 14). In Amniotic Fluid 9 (length of embryo 20 cm.) there was 1 unit of complement for goat erythrocytes in 0.9 cc of fluid, while the same amount gave (+) for sheep and (0) for guinea-pig and rabbit erythrocytes. In Amniotic Fluid 1 (length of embryo 30 cm.) 1 unit of complement for guinea-pig and rabbit erythrocytes was found in 0.1 cc of fluid, while 0.5 cc gave (+) for sheep and (0) for goat erythrocytes. Amniotic fluids are practically destitute of complement.

Table 3 gives the lysin titer of 19 amniotic fluids. The fluid from the youngest embryo contained no lysins and that of the most developed contained the greatest amount.

Polano² found that antitoxin was transmitted to the fetus from maternal blood but concluded that it was not transmitted to the amniotic fluid; to explain his results he advanced the theory of selective secretory action of the amniotic epithelium; on close inspection of his tables, however, we find that a small amount of antitoxin was present in the amniotic fluid as 0.4 c c neutralized the toxin mixed with it.

TABLE 3
LYSIN TITERS OF THE AMNIOTIC FLUIDS OF SWINE EMBRYOS

Number of Amniotic Fluid	Action of Amniotic Fluid on Various Erythrocytes			
	Sheep	Goat	Guinea-pig	Rabbit
25	0	0	0	0
10	0	0	+++ (0.1)	0
16	0	++	+++ (0.3)	+++
23	0	0	++	0
14	0	0	+++	0
9	++ (0.9)	+++	+++	0
18	+	++	+++ (0.3)	0
22	0	0	++	0
26	0	0	++	0
20	++	+	+++	0
12	0	+	+++ (0.3)	0
8	0	0	+++	0
17	+	++	+++	+++
13	+	+	+++	0
19	++	+++	+++ (0.3)	0
21	+	+	+++	0
11	0	0	+++	0
7	0	0	+++	0
15	+++	+++	+++ (0.1)	+++ (0.1)

Unless otherwise signified by figures in parenthesis the amount of amniotic fluid used was 0.5 cc; to this 1 unit of complement and erythrocyte suspension were added.

Goldmann³ found that pyrrhol blue when injected into a pregnant mouse was transmitted to the amniotic fluid, but not to the fetal serum; he said, however, that the stain was present in the skin and intestinal epithelium of the embryo; this, he claimed, was due to a direct taking up of the granules from the amniotic fluid by the skin and from swallowed amniotic fluid by the intestinal epithelium. It is not easy to understand Goldmann's conclusions as the amniotic epithelium has no blood supply separate from the fetal circulation, in fact, has no direct supply at all; there is always the possibility of a persistent chorio-amniotic connection to consider. Hirota,⁴ working with chick embryos,

¹ Zander, *J. Geburtsh. u. Gynäk.*, 1904, 53, p. 456.

² Vitale, *Pedagog.*, 1909.

³ *Ann. Coll. of Science of Tokyo*, 6, p. 4.

observed that there invariably was a sero-amniotic connection that becomes patent on the 11th day of incubation and permits a small amount of albumen to flow into the amniotic sac. However, this connection has never been demonstrated, I believe, in the mouse or swine embryos. Hence, the mechanism by which the pyrrhol blue granules obtain entrance into the amniotic sac and yet remain out of the fetal circulation is certainly obscure. Further work would seem necessary to make the matter clear.

The theory of the transudation of fluid direct from the maternal serum without entering the placental circulation is hardly worthy of further consideration when we recall that there are no stomata in the amnion except where it is in contact with the cord; Keibel and Mall⁵ quote Köster to this effect. If there was a transudation, should we not expect to find lysins in the earliest amniotic fluids as well as the later ones? Would there not be complement carried over in the transudate? My results show that these are not present.

If there is a transudation thru the amnion of the cord, as Williams⁶ suggests, as the principal origin of the amniotic fluid, we would expect to find at least a rough balance maintained between the antibodies of the fetus and the amniotic fluid. That this is true will be seen by a comparison of the lysin titers given in Tables 1 and 3. The remarkable similarity between them is highly suggestive of some relationship; the small differences are insignificant and to be expected when we remember that the balance is not maintained by instantaneous changes but must obtain by a relatively slow process. A comparison of Tables 2 and 3 demonstrates the wide disparity between the lysin content of the maternal serums and amniotic fluids and the extreme improbability of the latter being derived directly from the former. The almost complete absence of complement from the amniotic fluids may depend on its instability.

ALLANTOIC FLUIDS

The numbers of the allantoic fluids correspond to those of the embryos previously described.

Table 4 gives the complement and lysin titers. It is interesting to note that no complement was found in the fluids of the older embryos; in fact, Allantoic Fluid 24 from a 190 mm. embryo is the only one that contained it to any considerable degree. The lysins are present in greater amounts than complement even in the fluids of the younger

⁵ Human Embryology, 1910-1912.

⁶ Obstetrics, 1912.

embryos; at the bottom of the table, among the fluids of the older embryos, the lysins take on the semblance both qualitatively and quantitatively of the lysins of the embryo blood serums.

R. Neumann⁷ describes a dog in which the large intestine was cut off from the small intestine and the end tied; the free end of the small intestine was sutured to an artificial anus. After several months the secretion from the large intestine was collected and found to contain hemolysins qualitatively the same as those of the blood. This example indicates one method by which the antibodies of the allantoic fluid could originate. A comparison of the lysin titers of the allantoic

TABLE 4
FRESH ALLANTOIC FLUIDS OF SWINE EMBRYOS

Number of Allantoic Fluids	Complement Titers				Lysin Titers			
	Action of Allantoic Fluids 0.5 c c + 1 Unit of Amboceptor on Erythrocytes of				Action of Allantoic Fluids 0.5 c c + 1 Unit of Complement on Erythrocytes of			
	Sheep	Goat	Guinea-pig	Rabbit	Sheep	Goat	Guinea-pig	Rabbit
25	0	+++	0	0	+	+++	+++	0
16	++	0	0	0	+++	+++	+++	+++
24	+++	+++	+++	+++	+++	+++	+++	+++
18	0	0	0	0	+++	++	+++ (0.2 c c)	++
22	0	+	+++	0	0	0	0	0
26	0	+++	0	0	0	+++	0	0
17	0	0	0	0	+	0	+++	++
14	0	0	0	0	+	0	++	+
19	0	0	0	0	0	0	+	+
21	0	0	0	0	0	0	+++	0
11	0	0	0	0	+	++	+++ (0.1 c c)	+
15	0	0	0	0	0	0	+++ (0.05 c c)	+

The figures in parenthesis denote the amount of allantoic fluid used; otherwise 0.5 c c was used.

fluids with those of the embryonic serums shows their remarkable similarity to each other; there are some differences, it is true, as in Embryo 24, but this might be accounted for (1) in that nearly twice as much allantoic fluid was used as blood serum, and (2) there may be a constant pouring in of antibodies into the allantoic sac and by absorption of the water they become more concentrated. If this latter assumption be true, then why do we not have the greatest concentration in the oldest fluids? One explanation that could be offered is that in the swine fetus the allantoic fluid is relatively small in amount early in its development but later on the fluid increases relatively very much,

⁷ *Arch. u. H. pathol. anat. Inst.*, zu Tübingen, 1911, 7, p. 546.

this incoming fluid not only diluting the incoming antibodies but as the total amount is several times greater than it was in the earlier stages the amounts of stored antibodies become negligible; birth occurs shortly after the allantoic fluid reaches its maximum quantity and there is not much time for further concentration.

OPSONIC INDICES

Table 5 gives the opsonic index of the different serums and fluids for *Micrococcus aureus*, *Streptococcus pyogenes*, and *Bacillus subtilis*. A study of this table shows that the opsonins agree essentially with the lysins in their titers. In these tests the titers of several serums or fluids of about the same age were averaged, and this is the figure given in the table.

TABLE 5
OPSONIC INDICES

Length of Embryos, Cm.	Micrococcus Aureus			Streptococcus Pyogenes			Bacillus Subtilis: Fetal Serum
	Fetal Serum	Amniotic Fluid	Allantoic Fluid	Fetal Serum	Amniotic Fluid	Allantoic Fluid	
8-10	0.04	0.58	0.040	0.24	0.19	0.06	
14-19	0.13	0.4	0.74	0.58			0.10
20-24	0.04	0.58	0.83	0.30		0.10	0.20
25-28	0.49	0.74	0.30	0.58			0.30
30+	0.70			0.83			

The serum of mature swine was taken as a standard. Human leukocytes were used.

W. Busse⁸ found the average normal opsonic index of newly-born children to be 0.4 as compared with that of their mother; that of the amniotic fluid was 0.13; that of the fetal urine was 0.045. These results are all lower than mine. Tunnicliff,⁹ working with infant's blood, says that "at birth the opsonic power of the blood serum toward streptococci, pneumococci, and staphylococci is a little less than that of adult serum . . ." This statement agrees with my results, as I found the average opsonic index of the embryos just prior to birth to average 0.7 for staphylococci and 0.83 for streptococci.

CONCLUSIONS

In the youngest embryos complement and lysins are inappreciable. Opsonins were present but averaged only 0.04, as measured by the opsonic index. Complement and opsonins increase as the age of the fetus increases. Lysins do not appear to increase.

⁸ Gynäk. Rundschau, 1909.

⁹ Jour. Infect. Dis., 1910, 7, p. 706.

In the amniotic fluids complement is only occasionally found ; lysins and opsonins resemble closely those of the fetal serums. The conclusions of Polano and Goldmann from their work, respectively, on antitoxins and vital stains, that the amnion has a selective secretory action, seems to be unwarranted. The theory of the transudation of the amniotic fluid from the maternal serum is untenable. The amniotic fluid is probably derived almost exclusively under normal conditions as a transudation from the cord and as a secretion from the surface of the fetus.

In the allantoic fluids complement was found only in the younger embryos. Lysins are found more prevalent in the earlier fluids but to a small extent also in the later.

STUDIES ON SYNTHETIC MEDIUMS

I. STUDY OF THE CHARACTERISTICS OF SOME BACTERIA ON A SIMPLE SYNTHETIC MEDIUM

FLORENCE HULTON-FRANKEL, HELENE BARBER
AND
ELEANOR PYLE

*From the Department of Bacteriology, Columbia University, College of Physicians
and Surgeons, New York*

The study of the metabolism of bacteria has made very little progress until the present time, because it is impractical to study the end-products of any reaction without knowing the chemical composition of the raw materials. The basis of most bacterial culture mediums in the past has been beef infusion or extract, and peptone with the addition of agar to solidify it. It is usually stated that the agar does not add anything to the food of the bacteria, but merely gives a convenient neutral solid. This is not true. It has been found in this laboratory that *B. typhosus* will grow on a medium made of agar alone, which was previously extracted a number of times with water. Peptone is a mixture, obtained from the digestion of protein with pepsin and hydrochloric acid, and does not represent a chemical entity. In order, then, to attack the problem of bacterial metabolism, it is necessary to secure a culture medium of perfectly definite chemical composition, which can always be duplicated.

Uschinsky¹ had this in mind when he proposed his synthetic medium. Most synthetic mediums, since his time, have been some modification of his formula. However, it is possible to grow bacteria in a medium much simpler than Uschinsky's. Aside from the fact that the medium must contain sufficient nitrogen, carbon, etc., in a form in which they can be assimilated by the bacteria, the medium must be nearly isotonic with bacterial protoplasm and must have and maintain a favorable hydrogen-ion concentration.

It has been repeatedly pointed out that small variations in the hydrogen-ion concentration play a large rôle in biological processes. It has been shown in many researches on the action of enzymes that in order that satisfactory results be obtained, it is necessary to use solu-

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¹ Centralbl. f. Bakteriöl., I, O., 1893, 14, p. 346.

tions that are prepared with a view to preventing sharp variations in the hydrogen-ion concentration. The same holds true for the study of the growth of microbes, in whose metabolism it has been shown that enzymes have a prominent place. In these experiments, much attention has been given to preparing the culture medium so that it would prevent sharp changes in the hydrogen-ion concentration, and to this end a solution as heavily buffered as possible, consistent with osmotic requirements, was used. It is believed that this type of culture medium offers the most satisfactory means for the study of bacterial growth and metabolism, since it admits of prolonged growth without undue change in conditions. Especially is this necessary with organisms which have closely limited zones of optimum growth.

Clark and Lubs² in their recent work have indicated broadly the importance of these considerations. In working with synthetic medium of simple composition, Doryland³ apparently overlooked this aspect of the problem, for in his paper no attention is given to this phase of the work. It is true that his medium, neutralized to phenolphthalein ($pH=8.5$) contained about 15 gm. of mixed phosphates per liter, so that the solutions used by him were probably heavily enough buffered.

An attempt was made in these experiments to keep the osmotic pressure of the medium about that of mammalian blood serum, that is, isotonic with $M/6$ NaCl. The medium used contained alkali equivalent to 0.218 mols which, when neutralized with acid, yielded a salt concentration of 0.218 molar which when we remember that phosphates are only about 65% dissociated gives a solution almost isotonic with $M/6$ NaCl. In Doryland's experiments a much greater osmotic pressure was maintained. In his work he used a solution containing nearly 40 gm. of salts per liter which would probably be isotonic with $M/2$ or $M/3$ NaCl. It may probably be due to this unfavorable osmotic environment that many of the organisms he worked with did not grow.

A medium was prepared having the simplest composition consistent with these requirements. In the first experiments, the medium was made in hydrogen-ion concentrations varying from 10^{-5} - 10^{-8} N. It consisted of phosphoric acid, acetic acid, ammonia and potassium and sodium hydroxid in such proportions as to give the desired hydrogen-

² *Jour. Bacteriol.*, 1917, 2, p. 1, etc.

³ *Ibid.*, 1916, 1, p. 135.

ion concentration. The medium in its final form was of the following composition:

$$\left. \begin{array}{l} 129.5 \text{ c c of molar } \text{H}_3\text{PO}_4 \\ 18.8 \text{ c c of molar } \text{CH}_3\text{COOH} \\ 17.8 \text{ c c of molar } \text{NH}_4\text{OH} \end{array} \right\} \text{diluted to 500 c c.}$$

and to this was added 10 c c each of 0.01% CaCl_2 , 0.01% FeCl_3 and 0.01% MgSO_4 and sufficient 2 molar alkali (equal mols of NaOH and KOH) to give the proper hydrogen-ion concentration. The amounts used are given in Table 1.

TABLE 1
AMOUNTS USED IN THE PREPARATION OF THE MEDIUM

P _H	C c of Stock	C c of 2 Molar Alkali	C c Final Dilution
5	500	62.5	1,000
6	500	85	1,000
7	500	100	1,000
8	500	125	1,000

It was not feasible to make any lower hydrogen-ion concentrations, as when the solution becomes alkaline the phosphates are precipitated out. The solution containing the phosphoric, acetic acids, ammonia and alkali was diluted to 1,000 c c, after the addition of 10 c c each of 0.01% CaCl_2 , MgSO_4 and FeCl_3 . The medium was water clear, and after being tubed in sterile tubes was sterilized by steam on 3 successive days, for 15 minutes. The hydrogen-ion concentration was determined after each sterilization and in no case did it change.

This medium was inoculated with a group of organisms which were chosen as representatives of the different classes of bacteria as grouped in Chester's Manual. No streptococci were used, as it was not feasible to carry streptococci along with the other organisms. The organisms were planted at first merely with the idea of seeing how many would grow in a medium of this composition. The organisms were planted in duplicate by each of 2 workers, and one set of each was used for recording growth, while the other was used for determining the change in hydrogen-ion concentration. After 24 hours 3 loopfuls of the culture were placed on the surface of an agar slant and growth recorded as positive or negative after 24 hours. The 1 culture was incubated for a week just as it was, while the second one, after the transplant to agar was made, was divided into 2 portions — 1 was used for the hydrogen-ion concentration determination after 24 hours, while the second portion was incubated for 1 week, when transplants were again made and the hydrogen-ion concentration determined. The hydrogen-

ion concentration was obtained by adding Clark and Lubs² indicators to the cultures and comparing the colors obtained with a set of standards, prepared according to Sørensen, treated with the same indicators. To each culture was added at least 3 suitable indicators. The results of the experiments are found in Table 2.

TABLE 2
GROWTH ON SYNTHETIC MEDIUM

Name of Organism	Medium with a Reaction of 10 -5N					Medium with a Reaction of 10 -6N				
	Growth		Final PH		Growth after 8 Days	Growth		Final PH		Growth after 8 Days
	24 Hrs.	8 Days	24 Hrs.	8 Days		24 Hrs.	8 Days	24 Hrs.	8 Days	
1. <i>B. mucosus</i>	+	+	5.5	7	Slight	+	+	6.5	7	Heavy
2. <i>M. tetragenus</i>	—	—	5	5	—	—	—	6	6	—
3. <i>B. subtilis</i>	+	+	6	7	Slight	+	+	6.5	7	Slight
4. <i>B. coli communior</i>	+	+	6	7	Slight	+	+	6.5	7	Heavy
5. <i>B. pyocyaneus</i>	+	+	6	7	Moderate, pigment	+	+	6	7	Heavy, pigment
6. <i>Staph. pyogenes albus</i> ..	—	—	5	5	—	—	—	6	6	—
7. <i>B. enteritidis</i>	+	+	5.2	7	Heavy	+	+	over 6	7	Moderate
8. <i>B. prodigiosus</i>	+	+	5.2	6.5	Slight	+	+	over 6	7	Slight, pigment
9. <i>B. megatherium</i>	+	+	5.2	6	Slight	+	+	over 6	7	Slight
10. <i>M. candelans</i>	—	—	5	5	—	—	—	6	6	—
11. <i>B. fecalis alkaligenes</i> ...	+	—	5.2	6.5	—	+	+	over 6	7	Slight
12. <i>B. anthracoides</i>	+	+	5.2	6.5	Slight	+	+	over 6	7	Slight
13. <i>M. catarrhalis</i>	—	—	5	5	—	—	—	6	6	—
14. <i>B. enteritidis</i> —Gaertner	+	+	5.2	6	Slight	+	+	over 6	7	Slight
15. <i>Staph. pyogenes aureus</i>	—	—	5	5	—	—	—	6	6	—
17. <i>B. mirabilis</i>	+	+	5.2	7	Slight	+	+	7	7	Slight
18. <i>M. citreus</i>	+	+	6	7	Slight	—	+	7	7	Slight
19. <i>B. aerogenes</i>	+	+	5.2	7	Moderate	+	+	over 6	7	Moderate
20. <i>B. cloacae</i>	+	+	5.2	7	Heavy	+	+	7	7	Heavy
21. <i>B. hemorrhagicus</i>	—	—	5	5	—	—	—	6	6	—
23. <i>B. fluorescens liquefaciens</i>	+	+	5.2	7	Moderate	+	+	7	7	Moderate
24. <i>B. acidilactici</i>	+	+	5.2	7	Heavy	+	+	over 6	7	Heavy
25. <i>B. buccalis</i>	+	+	5.2	7	Slight	+	+	over 6	7	Slight
27. <i>B. cholerae</i>	+	+	5.2	7	Moderate	+	+	over 6	7	Moderate
28. <i>B. viridans</i>	+	+	5	7	Moderate	+	+	over 6	7	Moderate
29. <i>Sp. rubrum</i>	—	—	5	5	—	—	—	6	6	—
30. <i>B. dysentery</i> —Kruse....	+	+	5	7	Slight	+	+	over 6	7	Slight
31. <i>Map. cholerae</i>	+	+	5.2	7	Slight	+	+	over 6	7	Slight
32. <i>B. lactis aerogenes</i>	+	+	5.2	7	Moderate	+	+	over 6	7	Heavy
35. <i>M. roseus</i>	—	—	5	5	—	—	—	6	6	—
36. <i>Sarc. roseus</i>	—	—	5	5	—	—	—	6	6	—
37. <i>B. capsulatus</i>	+	+	5.2	7	Heavy	+	+	6	7	Heavy
38. <i>Sarc. lutea</i>	+	—	5.2	7	Moderate, produces pigment	+	+	over 6	7	Slight, pigment
39. <i>B. dysenteriae</i> —Shiga.	+	+	5.2	7	Slight	+	+	7	7	Slight
40. <i>B. erythrogenes</i>	—	—	5	5	—	—	—	6	6	—
41. <i>B. violaceus</i>	+	+	6	7	Slight, pigment	+	+	7	7	Heavy
42. <i>B. proteus vulgaris</i>
43. <i>B. typhosus</i>

Two more points remained to be proven before the statement could be made that the medium was a good medium on which to grow these organisms: (1) that the organism would remain alive for long periods in the medium; (2) that the organism after growing on the synthetic medium for long periods of time had the same characteristics as before.

TABLE 2—Continued
GROWTH ON SYNTHETIC MEDIUM

Medium with a Reaction of 10 ⁻⁷ N					Medium with a Reaction of 10 ⁻⁸ N				
Growth		Final PH		Growth after 8 Days	Growth		Final PH		Growth after 8 Days
24 Hrs.	8 Days	24 Hrs.	8 Days		24 Hrs.	8 Days	24 Hrs.	8 Days	
+	+	7	7	Heavy, flocculent sediment, greasy ring around sides of tube at top	+	+	8	8	Heavy
+	—	7	7	Moderate	—	—	—	—	—
+	+	7	7	Moderate, a slight turbidity persisting	+	+	8	8	Slight
+	+	7	7	Heavy, slight sediment, greasy growth around sides of tube at top	+	+	8	8	Moderate
+	+	7	7	Heavy, much sediment, pigment in 24 hours	+	+	8	8	Heavy, pigment
+	+	7	7	Slight	—	—	8	8	—
+	+	7	7	Heavy, slight sediment	+	+	8	8	Heavy
+	+	7	7	Moderate, turbidity evenly distributed, pigment	+	+	8	8	Slight
+	+	7	7	Heavy, marked turbidity, much sediment	+	+	8	8	Slight
—	—	7	7	—	—	—	8	8	—
+	+	7	7	Moderate, slight sediment	+	+	8	8	Heavy
+	+	7	7.5	Heavy, marked turbidity, sediment	+	+	8	8	Slight
—	—	7	7	—	—	—	8	8	—
+	+	7	7	Moderate, sediment	+	+	8	8	Slight
+	+	7	7	Slight	—	—	8	8	—
+	+	7	7	Moderate	+	+	8	8	Moderate
+	+	7	7	Moderate, pigment developed in 8 days	+	+	8	8	Slight
+	+	7	7	Moderate, much flocculent sediment, slight turbidity	+	+	8	8	Moderate
+	+	7	7	Heavy, pellicle	+	+	8	8	Heavy
—	—	7	7	—	—	—	8	8	—
+	+	7	7	Green pigment, heavy pellicle in 24 hours, slimy sediment	+	+	8	8	Moderate
+	+	7	7.5	Heavy, marked turbidity, slight sediment	+	+	8	8	Heavy
—	+	7	7	Moderate, slight turbidity	+	—	8	8	Slight in 24 hours, negative in 8 days
+	+	7	7	Moderate, moderate turbidity, some sediment	+	+	8	8	Heavy
+	+	7	7.5	Moderate, greenish pellicle in 24 hours, falls to bottom of tube	+	+	8	8	Heavy, pigment
+	+	7	7	Slight, slight sediment	+	—	8	8	Slight for 24 hours, negative at 8 days
+	+	7	7	Slight, flocculent sediment	+	+	8	8	Slight
+	+	7	7	Slight, slight sediment	+	+	8	8	Moderate
+	+	7	7	Heavy, much flocculent sediment	+	+	8	8	Heavy
+	+	7	7	Slight, produced pigment	—	—	8	8	—
+	+	7	7	Heavy, marked turbidity, produces pigment	—	—	8	8	—
+	+	7	7	Moderate, flocculent sediment, greasy ring around sides of tube at top	+	+	8	8	Heavy
+	+	7	7	Slight, pigment produced	+	—	8	8	Negative at 8 days, slight in 24 hours
+	+	7	7	Slight	—	—	8	8	—
+	+	7	7	Slight	—	—	8	8	—
+	+	7	7	Moderate, moderate turbidity, pigment	+	+	8	8	Moderate
+	+	7	7.5	Heavy, slimy sediment					
+	+	7	7	Slight turbidity, slight sediment					

In order to carry out these observations, the organisms were carefully studied before and after they were planted on the synthetic medium. Since the greater number of organisms grew on the medium having an initial hydrogen-ion concentration of 10^{-7} and also gave better growth on this medium, this concentration was used. The medium had the following composition:

129.5 cc of molar H_3PO_4	12.79 gm.	} or {	} diluted up to give one liter.
18.8 cc of molar CH_3COOH	1.13 gm.		
17.8 cc of molar NH_4OH	0.62 gm.		
100 cc of molar $NaOH$	4.0 gm.		
100 cc of molar KOH	5.6 gm.		
10 cc of 0.01% $FeCl_3$	0.001 gm.		
10 cc of 0.01% $MgSO_4$	0.001 gm.		
10 cc of 0.01% $CaCl_2$	0.001 gm.		

The medium, as made up, was made from stock solutions which had been standardized against standard acid and alkali, but as this is not feasible in all laboratories, the formula is also given so that the various acids and salts may be weighed out.

The organism was inoculated into 2 tubes of the synthetic medium, having this composition and a hydrogen-ion concentration of 10^{-7} N. The tubes were incubated for 24 hours and 3 loopfuls from 1 tube were placed on the surface of an agar slant. The second tube was divided into 2 portions, 1 portion being used to determine the hydrogen-ion concentration, the other portion being incubated for a longer time for the same purpose.

TABLE 3
SUMMARY OF GROWTH AT DIFFERENT INITIAL HYDROGEN-ION CONCENTRATION

Initial pH	No. Planted	No. Grew in 24 Hrs.	No. Grew in 8 Days	No Growth
5	36	26	24	10
6	36	25	26	10
7	38	34	34	3
8	36	25	23	10

The agar slant was incubated for 24 hours and then the culture was studied in the following manner:

1. Morphology: $\left\{ \begin{array}{l} \text{Staining.} \\ \text{Spore formation.} \\ \text{Flagella.} \\ \text{Capsules.} \end{array} \right.$

2. Biologic Characteristics:

(a) Plate cultures on agar.

Deep and surface colonies for form, size, margin, surface, texture, color and consistency.

(b) Agar tube culture.

Slants and streaks for same characters as colonies on plates.

TABLE 4
CHARACTERISTICS AFTER GROWTH ON SYNTHETIC MEDIUM

No.	Change after		
	24 Hours	8 Days	1 Month
1	Peptonization of milk complete 24 hours sooner than before growth on synthetic medium	Same as 24 hours	
2	Growth same as before	Did not grow	Did not grow
3	Coagulation; reduction; peptonization of milk complete in 3 days as compared to 6 before planting in synthetic medium	Same as 24 hours	Complete in 2 days
4	Coagulation of milk more rapid; peptonization complete in less time	Same as 24 hours	
5	Gelatin liquefied more slowly; peptonization of milk more rapid	Same as 24 hours	Same as 24 hours for milk and gelatin; pigment produced very slowly; thermal death point changed from 15-30 min. at 63 C.
6	Altho this organism should liquefy gelatin it did not till after it had grown on the synthetic medium	Same as 24 hours	Did not grow
7	Same as before	Same	Same
8	Pigment appeared more quickly on potato; milk action more rapid; gelatin liquefaction slower	Same as 24 hours	Same as 24 hours
9	Peptonization of milk more rapid; liquefaction of gelatin slower	Same as 24 hours	Same as 24 hours
11	Same as original	Same as the original culture	.
12	Peptonization of milk more rapid; liquefaction of gelatin slower	Same as 24-hour culture	
14	Same as the original culture in all respects (24, 8 days, 1 month)		
15	Same as the original culture		Did not grow
17	Same as the original culture in all respects (24, 8 days, 1 month)		
18	Same as the original culture in all respects (24, 8 days, 1 month)		
19	Same as the original culture in all respects (24, 8 days, 1 month)		
20	Milk formed acid and coagulated more quickly; original did not liquefy gelatin in 7 days; after growth on synthetic medium, liquefied in 3 days	Same as 24 hours	
23	Same as the original culture in all respects (24, 8 days, 1 month)		
24	Same as the original culture in all respects (24, 8 days, 1 month)		
25	Did not grow	Same as original culture in all respects	
27	Coagulation of milk and peptonization more rapid than original	Same as 24-hour culture	
28	Same as the original culture in all respects (24, 8 days, 1 month)		
30	Same as the original culture in all respects (24, 8 days, 1 month)		
31	Liquefaction of gelatin slower	Same as 24-hour culture	
32	Same as the original culture in all respects (24, 8 days, 1 month)		
36	Same as the original culture in all respects (24, 8 days, 1 month)		
37	Peptonization of milk more rapid	Same as 24-hour culture	
38	} Same as original culture in all respects		
39			
40			
41			
42			

(c) Potato cultures—being especially careful to note production of gas, odor or discoloration.

(d) Gelatin.

Both stabs and streaks—time of liquefaction especially being noted.

(e) Bouillon—turbidity, surface growth, odor and sediment.

(f) Litmus milk—reaction, coagulation, digestion of casein, splitting of lactose, reduction of litmus, odor.

3. Biochemical Factors:

(a) Production of enzymes.

Sugar splitting with formation of acid and gas, coagulating and diastatic.

(b) Production of indol and phenol.

(c) Production of acid or alkali.

(d) Reduction of nitrates to nitrites.

(e) Reduction of litmus.

4. Pathogenicity: Reported in another paper.

The organisms were studied, as stated, after growing on the synthetic medium for 24 hours, at which time transplants were made to agar. The hydrogen-ion concentration was determined on $\frac{1}{2}$ of the contents of another tube. At the end of 8 days, one transplant was made to agar and a second one from the same tube by transferring, with a sterile pipet, 0.1 c c of the culture to another sterile tube of the synthetic medium. The culture in this tube was transplanted thereafter every 4 days for a period of at least 1 month. The hydrogen-ion concentration was determined on the tube which had incubated for 8 days. At the end of the month, transplants were made to agar and the organisms studied culturally according to the scheme presented. In addition, thermal death points were determined on the organisms which had grown for a month on the synthetic medium. The changes which took place are given in Table 4.

From the results it is apparent that the organism grown on the synthetic medium had all the necessary elements for their growth. None of the characteristics were lost and in the case of the peptonization of milk the process seemed to be hastened by growth on synthetic medium. This may be merely a question of favorable hydrogen-ion concentration for the process; just as the retarding of the time of liquefaction of gelatin may be a question of unfavorable hydrogen-ion concentration. Whether or not these surmises are correct can only be proven by further work which is under way. The medium in its present form is suitable for the growth of most saprophytic organisms and also for some of the facultative parasitic organisms, and it is hoped that the medium can be slightly modified so that the obligate parasites will grow in it. A solid medium of known composition will be the next step in the investigation.

STUDIES ON SYNTHETIC MEDIUMS

II. SUGAR FERMENTATIONS IN SYNTHETIC MEDIUMS

FLORENCE HULTON-FRANKEL AND HELENE BARBER

From the Department of Bacteriology, Columbia University, College of Physicians and Surgeons, New York

It was found in the experiments already reported¹ that the members of the colon typhoid group grew very well on a synthetic medium of very simple chemical composition, and that after growing on this medium for a month still retained their sugar fermenting properties intact. The next step in the investigation then was to add the sugar directly to the synthetic medium, and grow the organisms on this.

The medium, the same as previously used, consisted of the following:

129.5 cc of molar H_3PO_4	}	diluted up to give 1 liter of medium.
18.8 cc of molar CH_3COOH		
17.8 cc of molar NH_4OH		
100 cc of molar NaOH		
100 cc of molar KOH		
10 cc of 0.01% Fe_2Cl_6		
10 cc of 0.01% MgSO_4		
10 cc of 0.01% CaCl_2		

Using this as a basis the 4 sugars used were added in amounts sufficient to give 1% sugar. The 4 sugars were dextrose, saccharose, lactose and man-nite. About 5-7 cc of medium was placed in a test tube containing a small capsule, after the tube had been sterilized. The medium had first had added in 1 case litmus as an indicator and in the other Andrade's indicator.² The medium was sterilized by steam on 3 successive days. It was sterilized for exactly 15 minutes from the time the burner of the sterilizer was lighted. exactly 15 minutes from the time the burner of the sterilizer was lighted. A medium of this composition has an initial hydrogen-ion concentration of $10^{-7.0}$.

The cultures used were 24-hour transplants, the synthetic medium being inoculated with a 1 mm. loopful of the culture. Four sets of each culture were planted at once: (1) synthetic containing Andrade's indicator; (2) synthetic containing litmus; (3) synthetic containing no indicator, to be tested for the hydrogen-ion concentration, and (4) sugar broth containing litmus. The cultures and controls of each kind of medium were incubated for 24 hours and readings as to the formation of acid and gas. Like readings were taken at 48 hours and 8 days.

The hydrogen-ion concentration was determined in the set of cultures containing no indicator. The tubes were filled so that each tube contained not less than 10 cc, as the hydrogen-ion concentration had to be determined at the end of 24 hours, which used up about one-half the culture and the other half was incubated for 8 days and the final hydrogen-ion concentration determined then. The hydrogen-ion concentration was determined by matching

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¹ Jour. Infect. Dis., 1919, 24, p. 9.

² Holman Jour. Infect. Dis., 1914, 15, p. 227.

against a series of phosphate standards made according to Sørensen using the Clark and Lub³ indicators together with methyl-red, methyl orange and phenolphthalein, and in no case was a hydrogen-ion concentration determined with the use of less than 3 indicators. In some cases when the indicators did not overlap sufficiently it was necessary to estimate, and this was the case in all cases recorded as $10^{-6.5n}$.

The litmus used was made from Merck's cube litmus and on the whole was unsatisfactory as it reduced very readily. The color could sometimes be restored by shaking. From this standpoint the Andrade's indicator was much more satisfactory. In the case of dextrose, only 3 of the 60 organisms tried failed to produce acid or gas when they did so in broth. In the few cases in which the organism took longer to reach the end reaction on the synthetic medium than it did on broth, the failure may have been due to the fact that the organism had been for a long time on agar and no effort was made to rejuvenate the organism before using, transplants being made directly from the agar to the synthetic sugar medium. This may also explain some of the failures on the broth where gas and acid formation were expected, but failed to appear. The difference of 24 hours in the formation of gas in the synthetic medium containing litmus and that containing Andrade's indicator has little significance, as one set was planted in the morning and the other in the afternoon, and the next day they were examined as quickly as possible and in cases in which only a few tiny bubbles of gas had formed they were recorded as simply forming acid.

The formation of acid and gas in saccharose coincided in the sugar broth and the sugar synthetic medium, except that in a few cases the formation was somewhat slower in the second. The same statement may be made of the organisms planted on the lactose and mannite synthetic medium. In the case of lactose there were 2 organisms that worked more quickly on the synthetic medium than on broth, while with the mannite 2 organisms produced gas in the synthetic medium that only produced acid in the broth.

It can readily be seen that a simple sugar medium like this has distinct advantages over a sugar broth, as it does away with the necessity of first freeing the meat infusion of sugar. This medium could be of special advantage in routine field work, as the materials can be carried in less space and the medium is much more easily made, since it could readily be put up in tablet form, and by dissolving these tablets in sterile distilled water a sterile medium could be obtained at once.

³ Jour. Bacteriol., 1917, 2, p. 1.

STUDIES ON SYNTHETIC MEDIUMS. II. SUGAR FERMENTATIONS IN SYNTHETIC MEDIUMS

TABLE 1.—FERMENTATIONS ON SYNTHETIC MEDIUM

Name	Dextrose								Saccharose								Lactose								Mannite									
	Synthetic				Broth				Hydrogen Ion	Synthetic				Broth				Hydrogen Ion	Synthetic				Broth				Hydrogen Ion							
	Litmus		Andrade		Litmus		Andrade			Litmus		Andrade		Litmus		Andrade			Litmus		Andrade													
	24 hr.	48 hr. 8 days	24 hr.	48 hr. 8 days	24 hr.	48 hr. 8 days	24 hr.	48 hr. 8 days	24 hr.	48 hr. 8 days	24 hr.	48 hr. 8 days	24 hr.	48 hr. 8 days	24 hr.	48 hr. 8 days	24 hr.	48 hr. 8 days	24 hr.	48 hr. 8 days	24 hr.	48 hr. 8 days	24 hr.	48 hr. 8 days	24 hr.	48 hr. 8 days	24 hr.	48 hr. 8 days	24 hr.	48 hr. 8 days				
1. <i>Enterococcus</i> (64).....	ag		ag		ag		5	5	ag		ag		ag		5.5	5.2	ag		ag		ag		6.8	5.2	ag		ag		ag		6.8	5.2		
2. <i>B. coli</i> (95).....	ag		ag		ag		7	5			ag		ag		7	7	ag		ag		ag		5.2	5.2	ag		ag		ag		6.8	5.2		
3. <i>B. coli</i> (44).....	a	ag		ag			7	5		ag	6		a	ag	72		7	7	ag		ag		5.2	5.2	a	ag	6	a	ag	6	6.8	5.2		
4. <i>B. coli</i> (104).....	ag		ag		ag		5.2	5.2			a	ag	6		7	7	ag		ag		ag		6.8	5.2	ag		ag		ag		6.8	5.2		
5. <i>B. coli communior</i> (144).....	ag		ag		ag		7	5	ag	r		ag		ag		6	6	ag		ag		ag		5.2	5.2	ag		ag		ag		6.8	5.2	
6. <i>B. coli</i> (46).....	—	ag		—	ag		7	6.5		ag	r		ag		6.5	6.2	ag		ag		ag		5.5	5.2	a	ag		ag		ag		6.8	5.2	
7. <i>B. coli</i> (325).....	—	ag	ag		ag		7	5.2					—	—	7	7	ag		ag		ag		5.2	5.2	ag		ag		ag		6.8	5.2		
8. <i>B. coli</i> (45).....	—	—	a	—	—	a	—	—					—	—	—	—	—	—	—	—	—	—	—	—	a	—	—	—	—	—	—	—		
9. <i>B. coli</i> (52).....	ag		ag		ag		6.2	5.2					—	—	7	7	ag		ag		ag		6	5	ag		ag		ag		6.8	5.2		
10. <i>B. coli communior</i> (109).....	ag		ag		ag		7	5	ag	r		ag		ag		6.5	6.5	ag		ag		ag		5.2	5.2	ag		ag		ag		6.8	5.2	
11. <i>B. coli</i> (71).....	ag		ag		ag		5	5	ag		ag		ag		5	5	ag		ag		ag		5.2	5	ag		ag		ag		6.8	5.2		
12. <i>B. coli</i> (57).....	a	ag		ag		a	—	—					—	—	7	7	ag	r	7		ag		6.8	5.2	ag		ag		ag		6.8	5.2		
13. <i>B. coli communior</i> (137).....	ag		ag		ag		5.2	5.2		ag	r		ag		ag		7	5.2	ag		ag		6.2	5.2	ag		ag		ag		6.8	5.2		
14. <i>B. coli</i> (178).....	ag		ag		ag		6.7	5					—	—	7	7.5	ag	r		ag		6.2	5.2	ag		ag		ag		6.8	5.2			
15. <i>B. communis</i> (125).....	a	—	a	—	—	—	5.2	5.2		ag		ag	6		—	—	a	—	—	—	—	—	5.2	5.2	a	—	ag	ag	—	—	6.2	5.2		
16. <i>B. communis</i> (139).....	—	—	—	—	—	—	7	7					—	—	7	7	—	—	—	—	—	—	7	7	—	—	—	—	—	—	—	—		
17. <i>B. communior</i> (123).....	ag	r	—	—	ag		7	6.5	ag		ag		ag		5.5	5.2	ag		ag		ag		6.8	5.2	ag		ag		ag		6.8	5.2		
18. <i>B. coli</i> (330).....	—	ag	6	—	ag	6	—	—	ag		ag		ag		ag	72	—	—	—	—	—	—	6.8	5.2	a	ag	ag	—	—	—	—	6.8	5.2	
19. <i>B. communis</i> (444).....	ag		ag		ag		5	5					—	—	7	7	ag		ag		ag		6.8	5.2	ag		ag		ag		6.8	5.2		
20. <i>B. anaerogenes</i> (179).....	a	ag	a	—	a	—	7	6.5					—	—	7	7	ag		ag		ag		6.5	5.2	ag		ag		ag		6.8	5.2		
21. <i>B. aerogenes</i> (127).....	a	ag	a	—	a	—	5.2	5.2	a	ag		ag		ag		5.2	5.2	ag		ag		ag		6.8	5.2	ag		ag		ag		6.8	5.2	
22. <i>B. aerogenes</i> (126).....	ag		a	ag	ag		5.2	5.2		ag		ag		ag		7	5.2	ag		ag		ag		6.8	5.2	ag		ag		ag		6.8	5.2	
23. <i>B. aerogenes</i> (136).....	ag	r	ag	ag	ag		6.5	6.5	ag	r		ag		ag		6.5	6.5	ag		ag		ag		6.8	5.2	ag		ag		ag		6.8	5.2	
24. <i>B. aerogenes</i> (448).....	ag		a	ag	ag		5.2	5.2		ag		ag		ag		7	7	a	—	—	—	—	7	6.5	ag		ag		ag		6.8	5.2		
25. <i>B. aerogenes</i> (120).....	ag		ag		ag		6.5	5.2		ag		ag		ag		7	6	—	ag	126	—	ag		7	6.5	ag		ag		ag		6.8	5.2	
26. <i>B. aerogenes</i> (378).....	ag		ag		ag		6.5	5.2	a	ag		ag		ag		7	5.2	a	ag		ag		6.8	6.5	ag		ag		ag		6.8	5.2		
27. <i>B. aerogenes</i> (245).....	ag	r	ag		ag	r	6.5	6.5	ag		ag		ag		6.5	6.5	ag		ag		ag		6.8	6.5	ag		ag		ag		6.8	5.2		
28. <i>B. aerogenes</i> (445).....	ag		ag		ag		5.2	5.2	ag		ag		ag		ag		5	5	ag		ag		5.2	5.2	ag		ag		ag		6.8	5.2		
29. <i>B. acid lactici</i> (243).....	ag		ag		ag		6.5	5					—	—	7	7	ag		ag		ag		5.2	5.2	ag		ag		ag		6.8	5.2		
30. <i>B. acid lactici</i> (131).....	ag		ag		ag		6.5	5					—	—	7	7	ag		ag		ag		5.2	5.2	ag		ag		ag		6.8	5.2		
31. <i>B. acid lactici</i> (559).....	ag		ag		ag		5	5					—	—	7	7	ag		ag		ag		5.2	5.2	ag		ag		ag		6.8	5.2		
32. <i>B. acid lactici</i> (446).....	—	ag	—	—	ag		5	5					—	—	7	7	ag		ag		ag		5.2	5.2	ag		ag		ag		6.8	5.2		
33. <i>B. acid lactici</i> (138).....	—	ag	—	—	—	—	5	5					—	—	7	7	ag		ag		ag		5.2	5.2	ag		ag		ag		6.8	5.2		
34. <i>B. acid lactici</i> (447).....	—	a	—	—	a	ag	7	5					—	—	7	7	—	—	ag	56	—	ag	56	—	7	6.5	ag	a	ag	ag	7	6.8	5.2	
35. <i>B. capsulatus</i> (40).....	ag		ag		ag		6.5	6.5	ag	r		ag		ag		6.5	6.5	ag		ag		ag		6.8	5.2	ag		ag		ag		6.8	5.2	
36. <i>B. capsulatus</i> (329).....	ag		ag		ag		7	6.5	ag	r		ag		ag		6.5	6.5	ag		ag		ag		6.8	5.2	ag		ag		ag		6.8	5.2	
37. <i>B. capsulatus</i> (468).....	—	—	—	—	ag	72	7	7					—	—	7	7	—	—	—	—	—	—	—	7	7	—	—	—	—	—	—	7	6.8	
38. <i>B. suis</i> (530).....	—	—	a	ag	ag		7	7					—	—	7	7	—	—	—	—	—	—	7	7	a	—	ag	—	—	—	—	7	6.8	
39. <i>B. pneumoniae</i> (343).....	—	ag	6	—	ag		7	7		ag		—	—	—	7	6.5	—	—	ag	56	—	ag	56	—	7	7	—	—	—	—	—	7	6.8	
40. <i>B. pneumoniae</i> (37).....	—	ag	6	—	ag		7	7		ag		—	—	—	7	7	—	—	ag	56	—	ag	56	—	7	7	—	—	—	—	—	7	6.8	
41. <i>B. pneumoniae</i> (241).....	—	ag	6	—	ag	72	7	5		ag		—	—	—	7	5.2	—	—	ag	120	—	ag	120	—	a	a	ag	7	6.5	ag		ag	7	
42. <i>B. dysenteriae</i> (783).....	a	—	a	—	a	—	7	6.5					—	—	7	7	—	—	—	—	—	—	6.8	5.2	ag		ag		ag		6.8	5.2		
43. <i>Pfeiffer's bac.</i> (240).....	ag		ag		ag		6.5	6.5	ag		ag		ag		6	6.5	ag		ag		ag		6.8	5.2	ag		ag		ag		6.8	5.2		
44. <i>B. fowl typhoid</i> (751).....	—	—	—	—	ag	72	7	7					—	—	7	7		—	—	—	—	—	7	7	—	—	—	—	—	—	—	7	6.8	
45. <i>B. cloacae</i> (450).....	—	ag	ag		ag		6.5	6.5	ag	r		ag		ag		6.5	6.5	ag		ag		ag		7	7	—	—	—	—	—	—	7	6.8	
46. <i>B. cloacae</i> (23).....	—	ag	ag		ag		6.5	6.5	ag		ag		ag		6.5	6.5	ag		ag		ag		6.8	5.2	ag		ag		ag		6.8	5.2		
47. <i>B. cloacae</i> (24).....	—	ag	ag		ag		6.5	6.5	ag		ag		ag		6.5	6.5	—	—	ag		ag		7	6.5	ag		ag		ag		6.8	5.2		
48. <i>B. cloacae</i> (238).....	—	ag	—	—	ag		6.5	6.5		ag		ag		ag		7	6.5	—	—	ag	120	—	ag	120	—	ag	7	6.5	ag		ag		6.8	5.2
49. <i>B. fecalis alkaligenes</i>	—	—	—	—	—	—	7	7					—	—	7	7	—	—	—	—	—	—	7	7	—	—	—	—	—	—	—	7	6.8	
50. <i>B. coli communior</i>	ag		ag		ag		5.2	5.2	ag		ag		ag		5	5	ag		ag		ag		6.8	5.2	ag		ag		ag		6.8	5.2		
51. <i>B. typhosus</i>	a	ag	a	ag	a	—	7	6.5					—	—	7	7	—	—	—	—	—	—	7	7	—	—	—	—	—	—	7	6.5		
52. <i>B. dysenteriae</i> Shiga (196).....	—	—	—	—	—	—	7	7					—	—	7	7	—	—	—	—	—	—												

STUDIES ON SYNTHETIC MEDIUMS

III. SOME ANIMAL EXPERIMENTS WITH ORGANISMS GROWN ON SYNTHETIC MEDIUM

FLORENCE HULTON-FRANKEL AND ELEANOR PYLE

*From the Department of Bacteriology, Columbia University, College of Physicians
and Surgeons, New York*

A number of organisms were found that grew on a synthetic medium of the following composition:

129.5 cc of molar H_3PO_4	} diluted up to give one liter of medium
18.8 cc of molar CH_3COOH	
17.8 cc of molar NH_4OH	
100 cc of molar NaOH	
100 cc of molar KOH	
10 cc of 0.01% Fe_2Cl_3	
10 cc of 0.01% MgSO_4	
10 cc of 0.01% CaCl_2	

The medium, a water clear liquid having a hydrogen-ion concentration of 10^{-7}N , was tubed, and sterilized in steam on 3 successive days for 15 minutes. The organisms that grew in this medium did not change in their cultural and biochemical reactions after periods of growth varying from 1 day to 1 month. The object of this investigation was to find out if some of these organisms which were culturally unchanged, had changed in their behavior toward animals.

The organisms chosen were *B. mucosus* (Friedländer bacillus), *Microspira cholerae*, and *B. typhosus* (Hopkins' strain). The first two were tried for virulence and the typhoid for the production of antibodies.

A culture of *B. mucosus* was used which had grown in the synthetic medium for a period of 19 days, having been transplanted every 4 days, by transferring 0.1 cc of the old culture in a sterile pipet, to a new tube of synthetic medium. A transplant was made from this culture on agar and after 24 hours was washed off with sterile salt solution, and 0.5 cc of this, representing 0.05 of an agar slant, was injected intraperitoneally into a mouse at 11 a. m.; and the mouse, though still alive at 5 p. m., was found dead at 9 the next morning. The culture was recovered from the peritoneum and heart, the organisms having all the characteristics of the original culture, possessing a more marked capsule than the original culture.

Two other mice were inoculated with this culture—one directly from the synthetic culture. One mouse was given 1 cc of a synthetic culture, which had been transplanted several times, at 4 p. m. At the same time, another

mouse was inoculated with some of the centrifugate of the same culture, washed and resuspended in salt solution to the original volume. One cc of this was injected intraperitoneally. Both mice were found dead at 9 the next morning, and as before, the organism was recovered from the peritoneal cavity and from the heart.

Of the culture of *Microspira cholerae* used, 0.05 of an agar slant would kill a rabbit weighing 1,885 gm. in less than 18 hours. The culture was then grown in the synthetic medium for several transplants, 4 days apart, and then transplanted to agar. This agar slant was emulsified in sterile salt solution and injected intravenously in a rabbit. This was done in three rabbits without any effect.

The results given by the two organisms would lead us to suppose that *B. mucosus capsulatus* had not changed in virulence while the *Microspira cholerae* had. Just why this should be so, is not apparent at present, but future work may throw light on the subject. Whether the presence of the capsule on *B. mucosus* is instrumental in preserving its virulence is a matter of conjecture.

TABLE 1
DETAILS OF METHODS EMPLOYED IN IMMUNIZING THE RABBITS

Rabbit Number	Weight in Gm.	Number of Injection	Amount of Injections, Slant	Date of Injections	Culture
50	1,875	1	0.02	5/ 4/18	Original
	2,020	2	0.1	5/ 9/18	Original
	1,950	3	0.1	5/13/18	Original
	1,905	4	0.1	5/18/18	Original
	1,885	5	0.2	5/23/18	Original
97	1,610	1	0.02	4/31/18	Synthetic
	1,699	2	0.10	5/ 4/18	Synthetic
	1,660	3	0.10	5/ 9/18	Synthetic
	1,630	4	0.10	5/15/18	Synthetic
	1,690	5	0.5	5/18/18	Synthetic
26	2,460	1	0.02	4/31/18	Synthetic
	2,270	2	0.05	5/ 4/18	Synthetic
	2,280	3	0.10	5/ 9/18	Synthetic
	2,170	4	0.10	5/15/18	Synthetic
	2,210	5	0.5	5/18/18	Synthetic

The experiments with the typhoid bacillus were for the purpose of determining whether or not agglutinins were produced by the organism after it had grown on the synthetic medium, and if so, whether the serum produced would agglutinate the original organism and whether a serum produced by the original organism would agglutinate the organism grown in the synthetic medium.

Three rabbits were immunized, two with an organism grown in the synthetic for a week and kept on the synthetic medium until used, being transferred to agar 24 hours before the injection was to be given. The third one was immunized with the original culture.

One week after the last injection, the rabbits were bled from the ear in order to procure just enough blood to test the titer of the serum with the result outlined in Table 2.

TABLE 2
RESULTS OBTAINED IN TESTING THE TITER OF THE SERUM

Rabbit No.	Culture	Dilutions of Serum										Control
		1:20	1:500	1:2,000	1:8,000	1:40,000	1:60,000	1:80,000	1:100,000	1:120,000	1:130,000	
36	Orig.	+++	+++	++	++	+	+	+	+	±	—	—
36	Synth.	+++	+++	++	++	+	+	+	+	±	—	—
50	Orig.	+++	++	++	+	+	+	+	—	—	—	—
50	Synth.	+++	++	++	++	+	+	+	—	—	—	—
97	Orig.	+++	+++	++	++	+	+	+	+	—	—	—
97	Synth.	+++	+++	++	++	+	+	+	+	—	—	—

That the rabbits should have developed serums of such a high titer is undoubtedly due in part to the fact that the organism was injected without killing, but must be due largely to the fact that the rabbits themselves possessed a marked resistance to typhoid bacilli, as I have immunized rabbits before with much larger doses of live typhoid and did not get serum of such high titer. The table shows that the serums produced by immunizing the rabbit with the original typhoid possesses as marked agglutinating powers for the synthetic organism as it does for the original with which it was immunized, while the reverse is true, the serum produced with the synthetic organism agglutinates the original just as well.

The only conclusion that we can draw is that the synthetic medium is suitable for growth of *B. mucosus*, not changing its biologic or cultural characteristics or its pathogenicity, while it apparently lacks something necessary to produce the substance responsible for the pathogenicity of *Microspira cholerae*. It might be possible to modify the medium slightly and produce the substance. However, it may be of advantage to be able to attenuate cholera in this manner. *B. typhosus* seems to find in medium all the essentials for its metabolism, remaining at the end of a month's growth on the synthetic culturally, biologically and immunologically the same.

PARTIAL TENSION STREPTOCOCCI AND VACCINE PREPARATION¹

WADE W. OLIVER AND ORMAN C. PERKINS

From the Department of Bacteriology, Hoagland Laboratory, Long Island College Hospital, Brooklyn, New York

Although the respiration of bacteria has been especially reviewed by Kruse² and Meyer,³ yet by only a relatively few workers has attention been directed to the oxygen tension requirements of parasitic bacteria.

In this country, Rosenow's work on streptococci isolated from appendicitis, rheumatic joints, etc., and from glands draining the involved joints in arthritis deformans, is specially pertinent to the present discussion. He employed deep tubes of broth and agar. Of cultures from rheumatic joints, he says: "That the oxygen requirement is the chief factor to explain this difference in my results and the negative results of others is indicated also by the fact that the colonies never developed above 0.5 cm. from the top and never below 2 cm. from the bottom of the agar tubes. The largest number of colonies developed between 1.5 cm. from the top and 3.5 cm. from the bottom."⁴ Of cultures obtained from glands draining the lesions of arthritis deformans, he says: "All the streptococcal forms isolated have shown a marked preference for anaerobic conditions of growth in the primary culture."⁵ Further, he states: "It would seem as if in arthritis deformans the micro-organisms are taken up from the circulation by the endothelial cells which proliferate freely so that eventually the blood supply is reduced or cut off, in consequence of which there result areas of lowered oxygen tension, diminished nutrition and atrophy. Such conditions would favor the growth of organisms which on isolation are sensitive to oxygen."

As emphasized in a previous article,⁶ we have felt that the success-

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¹ Oliver: Preliminary Note, Long Island Med. Jour., 1918, 12, p. 132.

² Allg. Mikrobiologie, 1910.

³ Centrallbl. f. Bakteriol. I, O., 1909, 49, p. 305.

⁴ Jour. Infect. Dis., 1914, 14, p. 62.

⁵ Jour. Am. Med. Assn., 1914, 62, p. 1146.

⁶ Wherry and Oliver Jour. Infect. Dis., 1916, 19, p. 288.

ful cultivation of many known micro-organisms and of some of the unknown viruses of infectious diseases may be dependent more on the proper oxygen tension in the cultures than on the composition of the medium.

During the routine bacteriologic examinations at this laboratory within the past 12 months we have isolated from a variety of disease processes in the human body 32 strains of partial tension streptococci. Of these, 6 strains were isolated only by means of the partial tension method,⁶ aerobic and anaerobic cultures remaining sterile, and being discarded after from 5-15 days of incubation. The remaining 26 strains all yielded a decidedly more marked, and in certain cases luxuriant, growth in partial tension cultures than in aerobic and anaerobic cultures. In fact, the relative luxuriance of growth of many streptococci at partial tension is most striking.

TABLE 1
SOURCES AND OTHER DATA REGARDING THE STRAINS ISOLATED

Source of Streptococci	Number of Strains	Growth Only at Partial Tension	Vaccines Prepared
1. Sputum (asthma)	7	1	7
2. Pyorrhea	5	1	5
3. Mastoiditis	1
4. Throat	3
5. Furunculosis	3	..	3
6. Empyema	1	..	1
7. Uterus	8	2	..
8. Stool	1	..	1
9. Urine	1
10. "Cold Abscess"	2	2	..
Total	32	6	17

TECHNIC

1. *Sputum*.—In 7 cases suffering from marked asthma, streptococci were isolated in considerable numbers from the sputum. In 1 case, growth of streptococci was obtained only in the partial tension culture and in the remaining 6 cases growth was decidedly more marked under partial tension conditions than in aerobic or anaerobic cultures. Cultures were made as follows: Small masses of sputum were given 3 consecutive washings in sterile normal salt solution, after which they were streaked by means of a sterile glass spreader across human blood-agar (+1) plates. Partial tension conditions were supplied by incubating the plates at 37 C. in a sealed jar along with 3-5 plain agar (+1) slants inoculated with *B. subtilis* (Nowak).⁷ After 24-48 hours incubation, transplants from single colonies were made to slants of plain agar (+1) and dextrose agar (+1) and made partial tension by tightly connecting and incubating them at 37 C. with a plain agar (+1) slant of *B. subtilis*. By this method, the incubation of 4-6 tubes yielded sufficient growth within 24-48 hours to allow of the preparation of a vaccine. In no case was sufficient growth obtained on 3 days' incubation under aerobic and anaerobic conditions to admit of vaccine preparation.

⁷ Ann. de l'Inst. Pasteur, 1908, 22, p. 541.

2. *Pyorrhea*.—In 1 of the 5 cases a streptococcus was isolated only by means of partial tension cultures (plain agar (+1) and human blood agar (+1), no growth being obtained on these mediums under aerobic and anaerobic conditions. This case was one of severe pyorrhea for which rapid preparation of an autogenous vaccine was urgently requested. The partial tension method yielded a pure culture of a streptococcus which grew so well on plain agar (+1) that sufficient growth was obtained from 4 slants on 24 hours' incubation at 37 C. to allow of the preparation of a vaccine.

After preliminary swabbing of the gums with 95% alcohol, a small quantity of pus was expressed and obtained on a platinum loop. Plates were inoculated by the streak method. Isolation was effected by incubation at 37 C. of plain agar (+1) plates and human blood-agar (+1) plates made partial tension by the method of Nowak. After 24 hours' incubation, transplants of single colonies were made to partial tension slants of plain agar (+1) and dextrose agar (+1). In all 5 cases, vaccines were prepared from the growth obtained under diminished oxygen tension.

3. *Mastoiditis*.—In this case, pus obtained from the mastoid cells at operation was streaked in small quantities across plates of plain agar (+1) and human blood agar (+1). Both aerobic and anaerobic plates, when incubated at 37 C. for 24 hours, yielded growth. However, whereas in such cultures the growth consisted of isolated, tiny colonies, the growth obtained at partial tension, both on isolation and on subsequent transplants, was rather heavy and confluent.

Repeated transplants of the aerobic strain on plain agar every 24-48 hours results, after a number of transplants, in a more marked growth. The same applies to successive aerobic transplants made from the partial tension strain. This loss or modification of the oxygen tension requirement has frequently been noted. In reference to streptococci isolated from rheumatic joints, Rosenow⁸ states that "after cultivation from 1-8 months, the capacity to grow at a low temperature, the sensitiveness to oxygen, the excessive production of acid in dextrose broth, and the simultaneous affinity for joints, endocardium and myocardium are found to have largely or entirely disappeared."

We have very recently encountered an example of what we believe to be a complete and rather sudden modification of the oxygen requirement of a streptococcus within the body. Pus obtained from a cerebellar abscess in a young child showed, in direct smears, scattered gram +, tiny streptococci. Repeated smears were negative for acid-fast bacteria. Blood-agar plates were streaked with small quantities of the pus, and incubated at 37 C. aerobically and anaerobically and at partial tension. The aerobic and partial tension plates remained sterile, but anaerobically a considerable number of tiny, gray colonies of streptococci were obtained which failed to grow on aerobic and partial tension transplant. Eight days later, following operation,

⁸ Jour. Infect. Dis., 1914, 14, p. 66.

another specimen of pus was obtained. Direct smears showed enormous numbers of very small gram + streptococci. Aerobic cultures on human blood agar (+ 1), incubated for 24 hours at 37 C. revealed a few small colonies of streptococci and scattered colonies of staphylococci and a gram + bacillus. Six days later, a third specimen of pus when streaked on blood-agar (+ 1) plates yielded, on 24 hours' aerobic incubation at 37 C., a marked growth of streptococci. Macroscopically and microscopically, these streptococci were indistinguishable from the micro-organisms obtained in the first culture only under strict conditions of anaerobiosis. Although we realize that this is not an absolute criterion of the identity of the 2 strains, yet it would seem that the fact that in the second culture made 8 days after the original culture and following operation a few scattered colonies of streptococci were obtained on aerobic blood-agar plates points to a beginning change in the oxygen tension requirement of the previously sealed up micro-organism. Interpreted in this light, the marked aerobic growth obtained after another 6 days points to a most rapid adaptation of the streptococcus within the body to the altered oxygen environment obtained after operation and drainage.

4. *Throat*.—The 3 throat cultures were obtained from patients who were suffering from a moderately severe puerperal infection. These strains yielded a fairly marked growth aerobically on human blood-agar (+ 1) plates incubated at 37 C. and a very scant growth anaerobically. The growth on human blood agar (+ 1) at partial tension was somewhat greater than that obtained on aerobic incubation, but the difference in amount of growth was not so great as in the other cases reported in this article.

Swab cultures were taken from the tonsillar area of the throat and streak cultures made on human blood-agar (+ 1) plates, incubated at 37 C. Nowak's method was employed in making the cultures partial tension. The anaerobic cultures were made by sealing the plates in a jar containing pyrogallic acid and potassium hydroxid.

5. *Furuncles*.—In the 3 cases of furunculosis, in which the partial tension method alone yielded sufficient growth to allow of the preparation of a vaccine, the surface of the boils was cleansed with bichlorid of mercury solution (1:1,000), followed by 95% alcohol. The lesion was then punctured with a sterile needle and the pus expressed. Human blood-agar plates streaked with a small quantity of pus were incubated at 37 C. The aerobic plates, on 24 hours' incubation at 37 C. yielded considerable numbers of colonies of *Staphylococcus pyogenes albus* and *aureus*, with a few, scattered, minute colonies of streptococci. The partial tension plates on 24 hours' incubation yielded larger colonies of the streptococcus in decidedly larger numbers. On transplant of single partial tension colonies to slants of dextrose agar (+ 1) incubated at 37 C., sealed along with slants of *B. subtilis*, sufficient growth was obtained from 5 tubes after 30 hours' incubation to allow of the preparation of vaccines.

6. *Empyema*.—A loopful of pus was streaked across 2 plates of human

blood agar (+1). A streptococcus was isolated in pure culture, the aerobic plate showing scattered colonies, whereas the plate at diminished oxygen tension showed a luxuriant growth of streptococci after 24 hours' incubation at 37 C. Transplants to plain agar slants, incubated at partial tension for 24 hours, yielded sufficient growth to allow of the preparation of a vaccine.

7. *Uterus*.—In 8 cases of uterine infection, a partial-tension streptococcus was isolated in pure culture. In 1 case growth was obtained only under conditions of diminished oxygen tension, and in the remaining 7 cases the marked growth at partial tension was in contrast to the rather delicate aerobic and anaerobic growth. The material for cultures was obtained by Dr. Beck by inserting a sterile glass tube, provided with a suction valve, into the uterus, and drawing into the tube the uterine secretion. The collecting tubes, wrapped in sterile towels, were immediately sent to the laboratory. The pus was removed from the tube by means of a small platinum loop and was streaked on human blood-agar (+1) plates with a sterile glass spreader.

Three of the patients, who suffered from a clinically similar, moderately severe uterine infection following childbirth and who were delivered with such extreme emphasis placed on asepsis as to practically exclude the possibility of outside infection, gave a history of influenza dating back from 1-4 months prior to delivery. From these patients a similar streptococcus was isolated from the uterus and from the throat.

One of the remaining 5 cases was that of a very severe chorea in a child. Material was obtained from the uterus in the manner previously described. An obligative partial tension streptococcus was isolated in pure culture on plates of Martin's ascitic agar (0.5),⁹ after 3 days' incubation at 37 C. Transplants of single colonies to human blood-agar (+1) slants incubated aerobically at 37 C. remained sterile, whereas transplants to slants of human blood agar (+1) incubated at 37 C. at partial tension yielded a rather luxuriant growth of streptococci.

8. *Stool*.—A loopful of stool was thoroughly suspended in 5 cc of sterile salt solution and 1 loopful of the suspension was spread on the surface of 2 human blood-agar (+1) plates. One plate was incubated aerobically at 37 C. and the other plate at 37 C at partial tension. On 24 hours' incubation, the partial tension plate yielded a large number of colonies of streptococci, whereas the aerobic plate revealed only a few, scattered, tiny streptococcus colonies.

9. *Urine*.—One loopful of sediment, obtained by centrifuging 5 cc of urine at moderate speed for 20 minutes, was streaked across human blood-agar (+1) plates. The results were identical with those obtained in the case of the stool culture (cf. par. 8).

10. "*Cold Abscess*."—The report deals with 2 cases of "psoas abscess" developing in patients that, clinically, were suffering from tuberculosis.

A. Direct smears and smears from the pus treated with antiformin failed to reveal the presence of acid-fasts. Smears from the undiluted pus stained with Loeffler's methylene blue and by Gram's method revealed a few, scattered, gram-positive, short-chained streptococci. Human blood-agar (+1) plates incubated aerobically and anaerobically at 37 C. remained sterile on 2 weeks' incubation. Plates and slants of plain infusion agar (+1) incubated at partial tension at 37 C. yielded within 24 hours a delicate growth of very small streptococcus colonies in pure culture. After 2 successive transplants, 2 days apart on plain agar (+1) at partial tension, a marked growth was obtained

⁹ Jour. Path. and Bacteriol., 1908, 15, p. 76.

whereas transplants on human blood agar (+1) and plain infusion agar (+1) incubated aerobically at 37 C. remained sterile or yielded only a few minute colonies.

B. Direct smears of the pus and smears made following the antiformin method were negative for acid-fasts and other recognizable bacteria. (A later specimen, treated with antiformin, revealed acid-fasts. A small quantity of the pus injected intraperitoneally into a 300 gm. guinea-pig caused death in about 5 weeks with general tuberculosis.)

A loopful of undiluted pus was planted into each of 2 tubes of plain infusion broth (+1) and on slants of plain infusion agar (+1). One tube of each medium was incubated aerobically at 37 C. and 1 tube of each series at 37 C. at partial tension. On 3 weeks' continuous incubation, the cultures remained sterile and were discarded.

At the same time the cultures were made, 1 loopful of undiluted pus was evenly distributed on the surface of a slant of plain infusion agar (+1). This culture was placed in a Novy jar along with 3 plain agar (+1) slants inoculated with *B. subtilis*. In the bottom of the jar was placed about 20 cc of a saturated solution of calcium hydroxid, to remove the respiratory carbon dioxid from the system. The jar was sealed with hard paraffin and tightly clamped, and placed in the incubator at 37 C. After 4 days' incubation, the jar was opened. The calcium hydroxid solution was cloudy and possessed a scum of calcium carbonate on the surface. The plain agar culture, when examined by indirect natural light, showed a large number of extremely minute, very translucent, grayish colonies, most numerous on the surface of the slant immediately above the water of syneresis. Microscopically, the colonies were seen to be composed of very minute, very difficultly staining, streptococci (cf. below).

The culture was again sealed in a Novy jar along with 5 freshly inoculated slants of *B. subtilis* and about 20 cc of calcium hydroxid solution and re-incubated at 37 C. At the expiration of 2 days, the colonies showed no increase in size or numbers. Transplants were made to plain infusion agar (+1), Martin's ascitic agar (0.5), Dorset's egg medium, human blood agar (+1) and plain broth. One series of transplants was incubated at 37 C. in a sealed jar along with 4 plain agar (+1) slants of *B. subtilis* and the other series was incubated at 37 C. along with 4 plain agar (+1) slants of *B. subtilis* and about 20 cc of saturated calcium hydroxid solution. All of the tubes remained sterile and after 5 weeks' continuous incubation they were discarded.

MORPHOLOGY AND BIOLOGY

The streptococci isolated from asthma, pyorrhea, of mastoiditis, furunculosis, empyema, stool, urine and the uterus (4), had the following points in common:

In 24-hour plain infusion-agar (+1) and plain infusion-broth (+1) cultures, the cocci appeared as gram-positive, short-chained streptococci, with a considerable number of diplococcic forms and, less frequently, small somewhat irregular clumps which stained readily with ordinary anilin dyes. On human blood agar (+1) and on plain infusion agar at partial tension, the discrete colonies appeared gray and translucent, as did they on these mediums when aerobic growth was obtained. In plain infusion broth (+1) a fine, grayish, granular sediment developed after 24-48 hours' incubation at 37 C. Uniformly, the growth obtained at partial tension on plain infusion agar (+1) and human blood agar (+1) was strikingly greater than that observed on the same mediums when incubated aerobically or anaerobically.

In the remaining cases, the morphology or biology of the streptococci was sufficiently distinctive to merit a special description.

Cold Abscesses.—CASE 1.—This streptococcus, isolation of which was rendered possible only by the partial tension method, appeared as a small, gram-positive nonacid fast, short-chained streptococcus, the single cocci averaging about 0.4-0.5 mikrons in diameter. Staining was easily effected with the ordinary anilin dyes. The adaptation of this strain to growth at partial tension is suggested by the fact that its isolation was effected only by incubation of the cultures under diminished oxygen tension, as well as by the fact that subsequent partial tension transplants yielded a moderately heavy growth, whereas aerobic transplants yielded, at best, only a few, tiny scattered colonies and, in some cases, no growth at all. Even at partial tension, however, the primary cultures were characterized by their relative delicacy of growth.

CASE 2.—The cultural requirements of the strain isolated from this case were unique, in that isolation was effected only by incubating the culture under conditions of partial oxygen tension plus the removal of the respiratory carbon dioxide. As will be recollected, the growth on plain agar under such conditions appeared as very minute, very translucent colonies, even difficultly visible when examined by indirect natural light. Growth appeared within 4 days' incubation at 37 C. Transplants failed to develop.

Smears from single colonies, when stained with Loeffler's methylene blue for 2 minutes, Gram's method, warm, dilute (1:20) carbolfuchsin for several hours, and by the Ziehl-Neelsen carbolfuchsin method, revealed considerable numbers of very minute, very feebly staining coccoid bodies, which took the stain so lightly that they were not visible when examined by strong, artificial illumination. When stained for 5 minutes with a saturated alcoholic solution of victoria blue (Grubler), the micro-organisms stained rather readily and appeared under the ordinary Leitz 1:12 oil immersion lens and No. 3 ocular as very minute cocci of rather constant size, and so small as to be but little above the limit of visibility. These bodies occurred as diplococci and in short chains of from 4-5 members. Comparison with a smear from a culture of the globoid bodies of poliomyelitis, obtained through the kindness of Dr. Noguchi, revealed that the cocci were of about the size of the minute forms of the globoid bodies described by Flexner and Noguchi¹⁰ and Rosenow.¹¹

¹⁰ Jour. Exper. Med., 1913, 18, p. 461.

¹¹ Jour. Infect. Dis., 1918, 22, p. 281.

Uterus (Chorea).—In this case, which was one of very severe chorea, an obligative partial tension streptococcus was isolated in pure culture from the uterus a few hours prior to death. The microbe was a minute, gram-positive, short-chained streptococcus, very slightly larger than the second strain isolated from a cold abscess. It was isolated on plates of Martin's ascitic agar (0.5) incubated at partial tension. Aerobic and anaerobic cultures remained sterile. The colonies were minute, gray and translucent and in the primary partial tension culture numbered only six to eight. Transplants to human blood agar (+1), made partial tension, yielded a more marked growth, whereas aerobic transplants to the same medium remained sterile. Recently, Quigley¹² has reported a series of 21 cases of chorea in which he isolated a delicately-growing short-chained streptococcus from both the blood and spinal fluid, and in 14 cases from either one or the other.

Puerperal Sepsis.—In these 3 cases, which followed in the wake of the widespread respiratory infections which swept New York City during the winter of 1917-1918, rather similar streptococci were isolated in pure culture from the uterus and in mixed culture from the throat. The organisms occurred as gram-positive, long-chained streptococci. In 24 hour plain and dextrose infusion-broth (+1) cultures, chains of 6-8 cocci occurred, but the predominant forms were long chains of from 12-30 or more members, a diplococcus grouping being often rather prominent in the chains.

In plain infusion broth, after 24-48 hours' incubation at 37 C., a gray flocculent growth was visible at the bottom and on the side of the test tube. The supernatant broth remained unclouded. All of the strains were bile-insoluble and failed to ferment inulin aerobically or at partial tension.

The uterine strains, on isolation, exhibited a more marked adaptation to growth at partial tension than did the strains isolated from the throat. The former, moreover, grew better anaerobically than aerobically, whereas the reverse was true in the case of the throat strains. However, all exhibited their most marked growth under conditions of diminished oxygen tension.

Blood cultures on these cases at various stages of the uterine infection remained sterile. The plates were incubated at 37 C. both aerobically and at partial tension.

¹² Jour. Infect. Dis., 1918, 22, p. 198.

VACCINES

In 17 of the 32 cases reported, vaccines were prepared from the growth obtained at partial tension. In none of the cases was sufficient growth obtained aerobically within 48 hours' incubation at 37 C. to allow of the preparation of a vaccine, whereas in all cases sufficient growth resulted at partial tension within 2 days and, in certain cases, within 24 hours, thus allowing of the preparation of an autogenous vaccine without delay. In certain of the cases, the factor of time in the preparation of the vaccine was of importance.

In 3 cases of pyorrhea and 3 cases of asthma the reaction and the clinical improvement in the patient was rather marked following the administration of vaccine. In each case, a 24-48 hour growth on plain agar (+ 1) or dextrose agar (+ 1) was employed. The initial dosage was 1 minim of vaccine (equivalent to 5-10 million streptococci), the injections being made subcutaneously at 3-day intervals. The dosage was gradually increased up to 12-15 minims.

Although it was not feasible to treat a parallel series of cases with a vaccine prepared from growth obtained aerobically, and although it is often extremely difficult, to say the least, to determine exactly what share in the clinical improvement of a patient is attributable to the vaccine, yet it is at least theoretically possible that the antigen obtained by cultivating certain micro-organisms at partial tension may be modified or different from the antigen obtained by growth aerobically.

SUMMARY

From a series of 32 cases of rather diverse nature partial tension streptococci were isolated. In 1 case of asthma, 1 of pyorrhea, 1 of chorea, 1 of uterine infection and 2 of "cold abscess" growth was obtained only at partial tension. In one of the cases of "cold abscess" isolation of a very minute streptococcus was effected only by growth in an atmosphere of diminished oxygen from which the respiratory carbon dioxid was removed. In the remaining 26 cases, the relative luxuriance of growth at partial tension was in contrast to the meager growth obtained aerobically and, in certain cases, anaerobically.

By the use of the partial tension method sufficient growth was obtained usually within 24 hours and never later than 48 hours, to allow of the preparation of an autogenous vaccine. In none of the 17 cases in which vaccines were prepared was sufficient growth obtained aerobically or anaerobically within 48 hours to warrant the preparation of a vaccine.

A COMPARISON OF SIX DIFFERENT ANTIGENS IN THE WASSERMANN REACTION

E. H. RUEDIGER

From the Pathological Laboratory of the Bismarck Hospital, Bismarck, N. D.

Because many different kinds of antigens are being used in the Wassermann reaction 6 different kinds of antigen were subjected to comparative study. The antigens studied were alcoholic extract of human heart muscle (A. E. H. H.); alcoholic extract of syphilitic fetal liver (A. E. S. F. L.); alcoholic extract of dog heart muscle (A. E. D. H.); acetone insoluble antigen of dog heart muscle (A. I. D. H.); alcoholic extract of sheep heart muscle (A. E. S. H.) and acetone insoluble antigen of sheep heart muscle (A. I. S. H.).

PREPARATION OF REAGENTS AND TECHNIC OF TESTS

The antigens used in these tests were prepared in accordance with the usual methods. Fat and fibrous tissue were cut away, blood was washed away, the lean heart muscle was finely minced and 100 gm. of minced muscle were extracted with 1,000 c.c. of absolute alcohol at 37 C., with daily shaking, for 2 weeks. The preparation was filtered through paper and, as precipitate was formed, enough alcohol was added to dissolve the precipitate and maintain a clear solution. Acetone insoluble antigen was prepared in accordance with the method of Noguchi as described by Kolmer.¹ The alcoholic extract of syphilitic fetal liver was purchased from the Wassermann Laboratory, Chicago; beyond that the source is entirely unknown to me.

In Test 1 the largest quantity of turbid antigen solution which was not anticomplementary was compared with a clear solution of the same strength regardless of its anticomplementary property. In all other tests the antigen solutions were turbid and were used in the largest quantities that were not anticomplementary.

All human serums were heated to about 56 C. for 30 minutes before they were tested and all except Portion A in Table 4 were glycerolated. Of the glycerol-serum mixture each test tube received 0.2 c.c. The nonglycerolated portion was mixed with an equal volume of salt solution and of this each test tube received 0.2 c.c.

As complement the mixed serum of 3 guinea-pigs were used in dilutions of 1:5, 1:10 and 1:20; 0.2 c.c. of diluted complement was used in each test tube.

Antihuman hemolytic amboceptor prepared on a rabbit was used exclusively. With glycerolated human serum the amboceptor was titrated in the presence of 50% glycerol corresponding to the serum-glycerol mixture used in the test. The smallest quantity which in the presence of 0.2 c.c. of 1:10 dilution of complement completely dissolved the test dose of corpuscles in

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¹ Infection, Immunity and Specific Therapy, 1917, p. 446.

1 hour was called 1 g unit and was used in the tests. For the nonglycerolated serums glycerol was not used in the titration of amboceptor, the smallest quantity which in the presence of 0.2 c c of 1:10 dilution of complement completely dissolved the test dose of corpuscles in 1 hour was called 1 unit and was used in testing the nonglycerolated serums.

The blood corpuscles used were a 2.5% suspension of washed human blood corpuscles and the test dose was 0.2 c c.

TABLE 1
TURBID ANTIGEN SOLUTION COMPARED WITH CLEAR ANTIGEN SOLUTION

Number of Serum	Kind of Antigen Solution	Antigen Dilution	Amboceptor per Tube Unit	Readings*						Results	
				Antigen Tubes			Control Tubes				
				1	2	3	1'	2'	3'		
1	Turbid	1:30	1 g	+	+	±	+	+	±	Negative,	—
2	Clear	1:30	1 g	+	+	±	+	+	±	Negative,	—
	Turbid	1:30	1 g	+	+	tr	+	+	tr	Negative,	—
3	Clear	1:30	1 g	+	+	tr	+	+	tr	Negative,	—
	Turbid	1:30	1 g	+	+	±	+	+	±	Negative,	—
4	Clear	1:30	1 g	+	+	±	+	+	±	Negative,	—
	Turbid	1:30	1 g	+	+	±	+	+	±	Negative,	—
5	Clear	1:30	1 g	+	+	±	+	+	±	Negative,	—
	Turbid	1:30	1 g	+	+	±	+	+	±	Negative,	—
6	Clear	1:30	1 g	+	+	tr	+	+	tr	Negative,	—
	Turbid	1:30	1 g	+	+	tr	+	+	tr	Negative,	—
7	Clear	1:30	1 g	+	+	tr	+	+	tr	Negative,	—
	Turbid	1:30	1 g	+	+	tr	+	+	tr	Negative,	—
8	Clear	1:30	1 g	+	+	±	+	+	±	Negative,	—
	Turbid	1:30	1 g	+	+	±	+	+	±	Negative,	—
9	Clear	1:30	1 g	+	+	±	+	+	±	Negative,	—
	Turbid	1:30	1 g	+	+	±	+	+	±	Negative,	—
10	Clear	1:30	1 g	+	+	±	+	+	±	Negative,	—
	Turbid	1:30	1 g	+	+	±	+	+	±	Negative,	—
11	Clear	1:30	1 g	+	+	±?	+	+	±	Faintly positive,	±
	Turbid	1:30	1 g	+	+	±	+	+	±	Negative,	—
12	Clear	1:30	1 g	tr	0	0	+	+	±	Strongly positive,	8+
	Turbid	1:30	1 g	+	tr	0	+	+	±	Strongly positive,	4+
13	Clear	1:30	1 g	+	tr	0	+	+	tr	Strongly positive,	3+
	Turbid	1:30	1 g	+	±	0	+	+	tr	Moderately positive,	2+
14	Clear	1:30	1 g	tr	0	0	+	+	±	Strongly positive,	8+
	Turbid	1:30	1 g	+	±	0	+	+	±	Strongly positive,	3+
15	Clear	1:30	1 g	tr	0	0	+	+	±	Strongly positive,	8+
	Turbid	1:30	1 g	+	+	tr	+	+	±	Weakly positive,	1+
16	Clear	1:30	1 g	+	tr	0	+	+	tr	Strongly positive,	3+
	Turbid	1:30	1 g	+	+	tr	+	+	tr	Negative,	—
17	Clear	1:30	1 g	+	±	0	+	+	±	Strongly positive,	3+
	Turbid	1:30	1 g	+	+	±	+	+	±	Negative,	—
18	Clear	1:30	1 g	+	tr	0	+	+	±	Strongly positive,	4+
	Turbid	1:30	1 g	+	+	±	+	+	±	Negative,	—
19	Clear	1:30	1 g	+	±	0	+	+	±	Strongly positive,	3+
	Turbid	1:30	1 g	+	+	±	+	+	±	Negative,	—
20	Clear	1:30	1 g	+	+	0	+	+	±	Moderately positive,	2+
	Turbid	1:30	1 g	+	+	±	+	+	±	Negative,	—

* In all tables 0 = no hemolysis; tr = hemolysis up to 50%; ± = hemolysis between 50% and 100%; + = complete hemolysis.

First incubation was in the refrigerator for 5 hours and second incubation in the incubator at 37 C. for 1 hour. The results were read about 3 hours after the corpuscles had been added.

TEST 1

When alcoholic antigen is slowly diluted with salt solution a turbid or opalescent solution results, while a small quantity of antigen added to a large quantity of salt solution forms a perfectly clear solution. In Test 1 twenty serums, 10 known negatives and 10 known positives, were tested with turbid

TABLE 2
ANTIGEN ADDED AFTER AND BEFORE THE COMPLEMENT

Number of Serum	Antigen Added	Antigen Dilution	Amboceptor per Tube Unit	Readings						Results	
				Antigen Tubes			Control Tubes				
				1	2	3	1'	2'	3'		
21	After	1:30	1 g	+	+	±	+	+	±	Negative,	—
22	Before	1:30	1 g	+	+	±	+	+	±	Negative,	—
	After	1:30	1 g	+	+	±	+	+	±	Negative,	—
23	Before	1:30	1 g	+	+	±	+	+	±	Negative,	—
	After	1:30	1 g	+	+	tr	+	+	tr	Negative,	—
24	Before	1:30	1 g	+	+	tr	+	+	tr	Negative,	—
	After	1:30	1 g	+	+	tr	+	+	tr	Negative,	—
25	Before	1:30	1 g	+	+	tr	+	+	tr	Negative,	—
	After	1:30	1 g	+	+	tr	+	+	tr	Negative,	—
26	Before	1:30	1 g	+	+	tr	+	+	tr	Negative,	—
	After	1:30	1 g	+	+	tr	+	+	tr	Negative,	—
27	Before	1:30	1 g	+	+	tr	+	+	tr	Negative,	—
	After	1:30	1 g	+	+	tr	+	+	tr	Negative,	—
28	Before	1:30	1 g	+	+	±	+	+	±	Negative,	—
	After	1:30	1 g	+	+	±	+	+	±	Negative,	—
29	Before	1:30	1 g	+	+	tr	+	+	tr	Negative,	—
	After	1:30	1 g	+	+	tr	+	+	tr	Negative,	—
30	Before	1:30	1 g	+	+	±	+	+	±	Negative,	—
	After	1:30	1 g	+	+	±	+	+	±	Negative,	—
31	Before	1:30	1 g	+	tr	0	+	+	tr	Strongly positive,	3+.
	After	1:30	1 g	+	tr	0	+	+	tr	Strongly positive,	3+.
32	Before	1:30	1 g	+	±	0	+	+	±	Strongly positive,	3+.
	After	1:30	1 g	+	±	0	+	+	±	Strongly positive,	3+.
33	Before	1:30	1 g	+	0	0	+	+	±	Strongly positive,	5+.
	After	1:30	1 g	+	0	0	+	+	±	Strongly positive,	5+.
34	Before	1:30	1 g	+	±	0	+	+	tr	Moderately positive,	2+.
	After	1:30	1 g	+	±	0	+	+	tr	Moderately positive,	2+.
35	Before	1:30	1 g	+	±	0	+	+	tr	Moderately positive,	2+.
	After	1:30	1 g	+	±	0	+	+	tr	Moderately positive,	2+.
36	Before	1:30	1 g	tr	0	0	+	+	±	Strongly positive,	8+.
	After	1:30	1 g	tr	0	0	+	+	±	Strongly positive,	8+.
37	Before	1:30	1 g	+	0	0	+	+	±	Strongly positive,	5+.
	After	1:30	1 g	+	0	0	+	+	±	Strongly positive,	5+.
38	Before	1:30	1 g	+	±	0	+	+	tr	Moderately positive,	2+.
	After	1:30	1 g	+	±	0	+	+	tr	Moderately positive,	2+.
39	Before	1:30	1 g	±	0	0	+	+	tr	Strongly positive,	5+.
	After	1:30	1 g	±	0	0	+	+	tr	Strongly positive,	5+.
40	Before	1:30	1 g	+	±	0	+	+	±	Strongly positive,	3+.
	After	1:30	1 g	+	±	0	+	+	±	Strongly positive,	3+.

and with clear antigen solution. The dilutions of the antigen were the same, 1:30.

Table 1 shows the results obtained with turbid and with clear antigen solutions on the 10 serums. Serums 1-10, inclusive, gave negative results with both antigen solutions. With Serums 11-20, inclusive, the turbid antigen solution gave much stronger positive results than did the clear antigen solution. Serum 11 gave \pm and a negative result; Serum 12 gave 8+ and 4+; Serum 13 gave 3+ and 2+; Serum 14 gave 8+ and 3+; Serum 15 gave 8+ and 1+; Serum 16 gave 3+ and a negative result; Serum 17 gave 3+ and a negative result; Serum 18 gave 4+ and a negative result; Serum 19 gave 3+ and a negative result; Serum 20 gave 2+ and a negative result.

TEST 2

In Test 2 twenty serums were tested by adding the antigen after and before the complement. Ordinarily I always mix the ingredients in the following order: (1) human serum; (2) complement, and (3) antigen. First incubation in the refrigerator 5 hours.

Whether antigen was added after or before the complement, the results, as shown in Table 2 were identical throughout.

TEST 3

Test 3 is a comparison of 6 different antigens. The antigens were: alcoholic extract of human heart (A. E. H. H.); alcoholic extract of syphilitic fetal liver (A. E. S. F. L.); alcoholic extract of dog heart (A. E. D. H.); acetone insoluble antigen of dog heart (A. I. D. H.); alcoholic extract of sheep heart (A. E. S. H.); acetone insoluble antigen of sheep heart (A. I. S. H.). Each antigen was made up in the form of a turbid solution and the largest quantity that was not anticomplementary was used as test dose.

Table 3 shows that the results obtained with the 6 different antigens varied greatly. Serums 41-76, inclusive, came from supposedly non-syphilitic persons. Serums 77-100, inclusive, came from syphilitics under treatment. Serums 41-61, inclusive, gave negative results with all of the 6 antigens. With Serums 62-76, inclusive, the results varied greatly, the supposed alcoholic extract of syphilitic fetal liver and the antigens prepared from dog heart gave positive results while the human antigen and with a few exceptions the sheep antigens gave negative results. With the known syphilitic serums the alcoholic extract of syphilitic fetal liver usually gave stronger positive results than did the alcoholic extract of human heart. As a rule, the alcoholic extract of dog heart gave stronger positive results than the alcoholic extract of human heart or of syphilitic fetal liver. The acetone

TABLE 3
SIX DIFFERENT ANTIGENS COMPARED

Number of Serum	Kind of Antigen	Antigen Dilution	Amboceptor per Tube Unit	Readings						Results	
				Antigen Tubes			Control Tubes				
				1	2	3	1'	2'	3'		
41	A. E. H. H.	1:30	1 g	+	+	±	+	+	±	Negative,	—.
	A. E. S. F. L.	1:40	1 g	+	+	±	+	+	±	Negative,	—.
	A. E. D. H.	1:30	1 g	+	+	±	+	+	±	Negative,	—.
	A. I. D. H.	1:30	1 g	+	+	±	+	+	±	Negative,	—.
	A. E. S. H.	1:30	1 g	+	+	±	+	+	±	Negative,	—.
	A. I. S. H.	1:30	1 g	+	+	±	+	+	±	Negative,	—.
42	A. E. H. H.	1:30	1 g	+	+	tr	+	+	tr	Negative,	—.
	A. E. S. F. L.	1:40	1 g	+	+	tr	+	+	tr	Negative,	—.
	A. E. D. H.	1:30	1 g	+	+	tr	+	+	tr	Negative,	—.
	A. I. D. H.	1:30	1 g	+	+	tr	+	+	tr	Negative,	—.
	A. E. S. H.	1:30	1 g	+	+	tr	+	+	tr	Negative,	—.
	A. I. S. H.	1:30	1 g	+	+	tr	+	+	tr	Negative,	—.
43	A. E. H. H.	1:30	1 g	+	+	tr	+	+	tr	Negative,	—.
	A. E. S. F. L.	1:40	1 g	+	+	tr	+	+	tr	Negative,	—.
	A. E. D. H.	1:30	1 g	+	+	tr	+	+	tr	Negative,	—.
	A. I. D. H.	1:30	1 g	+	+	tr	+	+	tr	Negative,	—.
	A. E. S. H.	1:30	1 g	+	+	tr	+	+	tr	Negative,	—.
	A. I. S. H.	1:30	1 g	+	+	tr	+	+	tr	Negative,	—.
44	A. E. H. H.	1:30	1 g	+	+	±	+	+	±	Negative,	—.
	A. E. S. F. L.	1:40	1 g	+	+	±	+	+	±	Negative,	—.
	A. E. D. H.	1:30	1 g	+	+	±	+	+	±	Negative,	—.
	A. I. D. H.	1:30	1 g	+	+	±	+	+	±	Negative,	—.
	A. E. S. H.	1:30	1 g	+	+	±	+	+	±	Negative,	—.
	A. I. S. H.	1:30	1 g	+	+	±	+	+	±	Negative,	—.
45	A. E. H. H.	1:30	1 g	+	+	±	+	+	±	Negative,	—.
	A. E. S. F. L.	1:40	1 g	+	+	±	+	+	±	Negative,	—.
	A. E. D. H.	1:30	1 g	+	+	±	+	+	±	Negative,	—.
	A. I. D. H.	1:30	1 g	+	+	±	+	+	±	Negative,	—.
	A. E. S. H.	1:30	1 g	+	+	±	+	+	±	Negative,	—.
	A. I. S. H.	1:30	1 g	+	+	±	+	+	±	Negative,	—.
46	A. E. H. H.	1:30	1 g	+	+	±	+	+	±	Negative,	—.
	A. E. S. F. L.	1:40	1 g	+	+	±	+	+	±	Negative,	—.
	A. E. D. H.	1:30	1 g	+	+	±	+	+	±	Negative,	—.
	A. I. D. H.	1:30	1 g	+	+	±	+	+	±	Negative,	—.
	A. E. S. H.	1:30	1 g	+	+	±	+	+	±	Negative,	—.
	A. I. S. H.	1:30	1 g	+	+	±	+	+	±	Negative,	—.
47	A. E. H. H.	1:30	1 g	+	+	±	+	+	±	Negative,	—.
	A. E. S. F. L.	1:40	1 g	+	+	±	+	+	±	Negative,	—.
	A. E. D. H.	1:30	1 g	+	+	±	+	+	±	Negative,	—.
	A. I. D. H.	1:30	1 g	+	+	±	+	+	±	Negative,	—.
	A. E. S. H.	1:30	1 g	+	+	±	+	+	±	Negative,	—.
	A. I. S. H.	1:30	1 g	+	+	±	+	+	±	Negative,	—.
48	A. E. H. H.	1:30	1 g	+	+	±	+	+	±	Negative,	—.
	A. E. S. F. L.	1:40	1 g	+	+	±	+	+	±	Negative,	—.
	A. E. D. H.	1:30	1 g	+	+	±	+	+	±	Negative,	—.
	A. I. D. H.	1:30	1 g	+	+	±	+	+	±	Negative,	—.
	A. E. S. H.	1:30	1 g	+	+	±	+	+	±	Negative,	—.
	A. I. S. H.	1:30	1 g	+	+	±	+	+	±	Negative,	—.
49	A. E. H. H.	1:30	1 g	+	+	tr	+	+	tr	Negative,	—.
	A. E. S. F. L.	1:40	1 g	+	+	tr	+	+	tr	Negative,	—.
	A. E. D. H.	1:30	1 g	+	+	tr	+	+	tr	Negative,	—.
	A. I. D. H.	1:30	1 g	+	+	tr	+	+	tr	Negative,	—.
	A. E. S. H.	1:30	1 g	+	+	tr	+	+	tr	Negative,	—.
	A. I. S. H.	1:30	1 g	+	+	tr	+	+	tr	Negative,	—.

TABLE 3—Continued
SIX DIFFERENT ANTIGENS COMPARED

Number of Serum	Kind of Antigen	Antigen Dilution	Ambo- ceptor per Tube Unit	Readings						Results	•
				Antigen Tubes			Control Tubes				
				1	2	3	1'	2'	3'		
50	A. E. H. H.	1:30	1 g	+	+	±	+	+	±	Negative,	—
	A. E. S. F. L.	1:40	1 g	+	+	±	+	+	±	Negative,	—
	A. E. D. H.	1:30	1 g	+	+	±	+	+	±	Negative,	—
	A. I. D. H.	1:30	1 g	+	+	±	+	+	±	Negative,	—
	A. E. S. H.	1:30	1 g	+	+	±	+	+	±	Negative,	—
	A. I. S. H.	1:30	1 g	+	+	±	+	+	±	Negative,	—
51	A. E. H. H.	1:30	1 g	+	+	±	+	+	±	Negative,	—
	A. E. S. F. L.	1:40	1 g	+	+	±	+	+	±	Negative,	—
	A. E. D. H.	1:30	1 g	+	+	±	+	+	±	Negative,	—
	A. I. D. H.	1:30	1 g	+	+	±	+	+	±	Negative,	—
	A. E. S. H.	1:30	1 g	+	+	±	+	+	±	Negative,	—
	A. I. S. H.	1:30	1 g	+	+	±	+	+	±	Negative,	—
52	A. E. H. H.	1:30	1 g	+	+	±	+	+	±	Negative,	—
	A. E. S. F. L.	1:40	1 g	+	+	±	+	+	±	Negative,	—
	A. E. D. H.	1:30	1 g	+	+	±	+	+	±	Negative,	—
	A. I. D. H.	1:30	1 g	+	+	±	+	+	±	Negative,	—
	A. E. S. H.	1:30	1 g	+	+	±	+	+	±	Negative,	—
	A. I. S. H.	1:30	1 g	+	+	±	+	+	±	Negative,	—
53	A. E. H. H.	1:30	1 g	+	+	±	+	+	±	Negative,	—
	A. E. S. F. L.	1:40	1 g	+	+	±	+	+	±	Negative,	—
	A. E. D. H.	1:30	1 g	+	+	±	+	+	±	Negative,	—
	A. I. D. H.	1:30	1 g	+	+	±	+	+	±	Negative,	—
	A. E. S. H.	1:30	1 g	+	+	±	+	+	±	Negative,	—
	A. I. S. H.	1:30	1 g	+	+	±	+	+	±	Negative,	—
54	A. E. H. H.	1:30	1 g	+	+	±	+	+	±	Negative,	—
	A. E. S. F. L.	1:40	1 g	+	+	±	+	+	±	Negative,	—
	A. E. D. H.	1:30	1 g	+	+	±	+	+	±	Negative,	—
	A. I. D. H.	1:30	1 g	+	+	±	+	+	±	Negative,	—
	A. E. S. H.	1:30	1 g	+	+	±	+	+	±	Negative,	—
	A. I. S. H.	1:30	1 g	+	+	±	+	+	±	Negative,	—
55	A. E. H. H.	1:30	1 g	+	+	tr	+	+	tr	Negative,	—
	A. E. S. F. L.	1:40	1 g	+	+	tr	+	+	tr	Negative,	—
	A. E. D. H.	1:30	1 g	+	+	tr	+	+	tr	Negative,	—
	A. I. D. H.	1:30	1 g	+	+	tr	+	+	tr	Negative,	—
	A. E. S. H.	1:30	1 g	+	+	tr	+	+	tr	Negative,	—
	A. I. S. H.	1:30	1 g	+	+	tr	+	+	tr	Negative,	—
56	A. E. H. H.	1:30	1 g	+	+	±	+	+	±	Negative,	—
	A. E. S. F. L.	1:40	1 g	+	+	±	+	+	±	Negative,	—
	A. E. D. H.	1:30	1 g	+	+	±	+	+	±	Negative,	—
	A. I. D. H.	1:30	1 g	+	+	±	+	+	±	Negative,	—
	A. E. S. H.	1:30	1 g	+	+	±	+	+	±	Negative,	—
	A. I. S. H.	1:30	1 g	+	+	±	+	+	±	Negative,	—
57	A. E. H. H.	1:30	1 g	+	+	±	+	+	±	Negative,	—
	A. E. S. F. L.	1:40	1 g	+	+	±	+	+	±	Negative,	—
	A. E. D. H.	1:30	1 g	+	+	±	+	+	±	Negative,	—
	A. I. D. H.	1:30	1 g	+	+	±	+	+	±	Negative,	—
	A. E. S. H.	1:30	1 g	+	+	±	+	+	±	Negative,	—
	A. I. S. H.	1:30	1 g	+	+	±	+	+	±	Negative,	—
58	A. E. H. H.	1:30	1 g	+	+	±	+	+	±	Negative,	—
	A. E. S. F. L.	1:40	1 g	+	+	±	+	+	±	Negative,	—
	A. E. D. H.	1:30	1 g	+	+	±	+	+	±	Negative,	—
	A. I. D. H.	1:30	1 g	+	+	±	+	+	±	Negative,	—
	A. E. S. H.	1:30	1 g	+	+	±	+	+	±	Negative,	—
	A. I. S. H.	1:30	1 g	+	+	±	+	+	±	Negative,	—

TABLE 3—Continued
SIX DIFFERENT ANTIGENS COMPARED

Number of Serum	Kind of Antigen	Antigen Dilution	Amboceptor per Tube Unit	Readings*						Results	
				Antigen Tubes			Control Tubes				
				1	2	3	1'	2'	3'		
59	A. E. H. H.	1:30	1 g	+	+	±	+	+	±	Negative,	—.
	A. E. S. F. L.	1:40	1 g	+	+	±	+	+	±	Negative,	—.
	A. E. D. H.	1:30	1 g	+	+	±	+	+	±	Negative,	—.
	A. I. D. H.	1:30	1 g	+	+	±	+	+	±	Negative,	—.
	A. E. S. H.	1:30	1 g	+	+	±	+	+	±	Negative,	—.
	A. I. S. H.	1:30	1 g	+	+	±	+	+	±	Negative,	—.
60	A. E. H. H.	1:30	1 g	+	+	±	+	+	±	Negative,	—.
	A. E. S. F. L.	1:40	1 g	+	+	±	+	+	±	Negative,	—.
	A. E. D. H.	1:30	1 g	+	+	±	+	+	±	Negative,	—.
	A. I. D. H.	1:30	1 g	+	+	±	+	+	±	Negative,	—.
	A. E. S. H.	1:30	1 g	+	+	±	+	+	±	Negative,	—.
	A. I. S. H.	1:30	1 g	+	+	±	+	+	±	Negative,	—.
61	A. E. H. H.	1:30	1 g	+	+	±	+	+	±	Negative,	—.
	A. E. S. F. L.	1:40	1 g	+	+	±	+	+	±	Negative,	—.
	A. E. D. H.	1:30	1 g	+	+	±	+	+	±	Negative,	—.
	A. I. D. H.	1:30	1 g	+	+	±	+	+	±	Negative,	—.
	A. E. S. H.	1:30	1 g	+	+	±	+	+	±	Negative,	—.
	A. I. S. H.	1:30	1 g	+	+	±	+	+	±	Negative,	—.
62	A. E. H. H.	1:30	1 g	+	+	tr	+	+	tr	Negative,	—.
	A. E. S. F. L.	1:40	1 g	+	+	0	+	+	tr	Weakly positive,	1+.
	A. E. D. H.	1:30	1 g	+	tr	0	+	+	tr	Strongly positive,	3+.
	A. I. D. H.	1:30	1 g	+	0	0	+	+	tr	Strongly positive,	4+.
	A. E. S. H.	1:30	1 g	+	+	tr	+	+	tr	Negative,	—.
	A. I. S. H.	1:30	1 g	+	+	tr	+	+	tr	Negative,	—.
63	A. E. H. H.	1:30	1 g	+	+	tr	+	+	tr	Negative,	—.
	A. E. S. F. L.	1:40	1 g	+	+	0	+	+	tr	Weakly positive,	1+.
	A. E. D. H.	1:30	1 g	+	+	0	+	+	tr	Weakly positive,	1+.
	A. I. D. H.	1:30	1 g	+	±	0	+	+	tr	Moderately positive,	2+.
	A. E. S. H.	1:30	1 g	+	+	tr	+	+	tr	Negative,	—.
	A. I. S. H.	1:30	1 g	+	+	tr	+	+	tr	Negative,	—.
64	A. E. H. H.	1:30	1 g	+	+	±	+	+	±	Negative,	—.
	A. E. S. F. L.	1:40	1 g	+	±	0	+	+	±	Strongly positive,	3+.
	A. E. D. H.	1:30	1 g	+	±	0	+	+	±	Strongly positive,	3+.
	A. I. D. H.	1:30	1 g	+	tr	0	+	+	±	Strongly positive,	4+.
	A. E. S. H.	1:30	1 g	+	+	±	+	+	±	Negative,	—.
	A. I. S. H.	1:30	1 g	+	+	±	+	+	±	Negative,	—.
65	A. E. H. H.	1:30	1 g	+	+	tr	+	+	tr	Negative,	—.
	A. E. S. F. L.	1:40	1 g	+	tr	0	+	+	tr	Strongly positive,	3+.
	A. E. D. H.	1:30	1 g	±	0	0	+	+	tr	Strongly positive,	5+.
	A. I. D. H.	1:30	1 g	0	0	0	+	+	tr	Strongly positive,	8+.
	A. E. S. H.	1:30	1 g	+	+	tr	+	+	tr	Negative,	—.
	A. I. S. H.	1:30	1 g	+	+	0	+	+	tr	Weakly positive,	1+.
66	A. E. H. H.	1:30	1 g	+	+	tr	+	+	tr	Negative,	—.
	A. E. S. F. L.	1:40	1 g	tr	0	0	+	+	tr	Strongly positive,	6+.
	A. E. D. H.	1:30	1 g	0	0	0	+	+	tr	Strongly positive,	8+.
	A. I. D. H.	1:30	1 g	tr	0	0	+	+	tr	Strongly positive,	6+.
	A. E. S. H.	1:30	1 g	+	+	0	+	+	tr	Weakly positive,	1+.
	A. I. S. H.	1:30	1 g	+	+	0	+	+	tr	Weakly positive,	1+.
67	A. E. H. H.	1:30	1 g	+	+	0	+	+	0	Negative,	—.
	A. E. S. F. L.	1:40	1 g	+	+	0	+	+	0	Negative,	—.
	A. E. D. H.	1:30	1 g	+	tr	0	+	+	0	Moderately positive,	2+.
	A. I. D. H.	1:30	1 g	+	0	0	+	+	0	Strongly positive,	3+.
	A. E. S. H.	1:30	1 g	+	+	0	+	+	0	Negative,	—.
	A. I. S. H.	1:30	1 g	+	+	0	+	+	0	Negative,	—.

TABLE 3—Continued
SIX DIFFERENT ANTIGENS COMPARED

Number of Serum	Kind of Antigen	Antigen Dilution	Amboceptor per Tube Unit	Readings						Results	
				Antigen Tubes			Control Tubes				
				1	2	3	1'	2'	3'		
68	A. E. H. H.	1:30	1 g	+	+	±	+	+	±	Negative,	—.
	A. E. S. F. L.	1:40	1 g	+	+	tr	+	+	±	Weakly positive,	1+.
	A. E. D. H.	1:30	1 g	+	tr	0	+	+	±	Strongly positive,	4+.
	A. I. D. H.	1:30	1 g	+	0	0	+	+	±	Strongly positive,	5+.
	A. E. S. H.	1:30	1 g	+	+	±	+	+	±	Negative,	—.
	A. I. S. H.	1:30	1 g	+	+	±	+	+	±	Negative,	—.
69	A. E. H. H.	1:30	1 g	+	+	±	+	+	±	Negative,	—.
	A. E. S. F. L.	1:40	1 g	0	0	0	+	+	±	Strongly positive,	10+.
	A. E. D. H.	1:30	1 g	0	0	0	+	+	±	Strongly positive,	10+.
	A. I. D. H.	1:30	1 g	±	0	0	+	+	±	Strongly positive,	6+.
	A. E. S. H.	1:30	1 g	+	+	±	+	+	±	Negative,	—.
	A. I. S. H.	1:30	1 g	+	+	±	+	+	±	Negative,	—.
70	A. E. H. H.	1:30	1 g	+	+	±	+	+	±	Negative,	—.
	A. E. S. F. L.	1:40	1 g	±	0	0	+	+	±	Strongly positive,	6+.
	A. E. D. H.	1:30	1 g	±	0	0	+	+	±	Strongly positive,	6+.
	A. I. D. H.	1:30	1 g	+	0	0	+	+	±	Strongly positive,	5+.
	A. E. S. H.	1:30	1 g	+	+	±	+	+	±	Negative,	—.
	A. I. S. H.	1:30	1 g	+	+	±	+	+	±	Negative,	—.
71	A. E. H. H.	1:30	1 g	+	+	±	+	+	±	Negative,	—.
	A. E. S. F. L.	1:40	1 g	+	±	0	+	+	±	Strongly positive,	3+.
	A. E. D. H.	1:30	1 g	+	±	0	+	+	±	Strongly positive,	3+.
	A. I. D. H.	1:30	1 g	+	+	0	+	+	±	Moderately positive,	2+.
	A. E. S. H.	1:30	1 g	+	+	±	+	+	±	Negative,	—.
	A. I. S. H.	1:30	1 g	+	+	±	+	+	±	Negative,	—.
72	A. E. H. H.	1:30	1 g	+	+	±	+	+	±	Negative,	—.
	A. E. S. F. L.	1:40	1 g	+	±	0	+	+	±	Strongly positive,	3+.
	A. E. D. H.	1:30	1 g	+	±	0	+	+	±	Strongly positive,	3+.
	A. I. D. H.	1:30	1 g	+	tr	0	+	+	±	Strongly positive,	4+.
	A. E. S. H.	1:30	1 g	+	+	±	+	+	±	Negative,	—.
	A. I. S. H.	1:30	1 g	+	+	±	+	+	±	Negative,	—.
73	A. E. H. H.	1:30	1 g	+	+	±	+	+	±	Negative,	—.
	A. E. S. F. L.	1:40	1 g	+	+	0	+	+	±	Moderately positive,	2+.
	A. E. D. H.	1:30	1 g	+	+	0	+	+	±	Moderately positive,	2+.
	A. I. D. H.	1:30	1 g	+	+	0	+	+	±	Moderately positive,	2+.
	A. E. S. H.	1:30	1 g	+	+	±	+	+	±	Negative,	—.
	A. I. S. H.	1:30	1 g	+	+	±	+	+	±	Negative,	—.
74	A. E. H. H.	1:30	1 g	+	+	±	+	+	±	Negative,	—.
	A. E. S. F. L.	1:40	1 g	+	+	±	+	+	±	Negative,	—.
	A. E. D. H.	1:30	1 g	+	+	±	+	+	±	Negative,	—.
	A. I. D. H.	1:30	1 g	+	+	0	+	+	±	Moderately positive,	2+.
	A. E. S. H.	1:30	1 g	+	+	±	+	+	±	Negative,	—.
	A. I. S. H.	1:30	1 g	+	+	±	+	+	±	Negative,	—.
75	A. E. H. H.	1:30	1 g	+	+	±	+	+	±	Negative,	—.
	A. E. S. F. L.	1:40	1 g	+	±	0	+	+	±	Strongly positive,	3+.
	A. E. D. H.	1:30	1 g	+	0	0	+	+	±	Strongly positive,	5+.
	A. I. D. H.	1:30	1 g	±	0	0	+	+	±	Strongly positive,	6+.
	A. E. S. H.	1:30	1 g	+	+	±	+	+	±	Negative,	—.
	A. I. S. H.	1:30	1 g	+	+	±	+	+	±	Negative,	—.
76	A. E. H. H.	1:30	1 g	+	+	±	+	+	±	Negative,	—.
	A. E. S. F. L.	1:40	1 g	±	0	0	+	+	±	Strongly positive,	6+.
	A. E. D. H.	1:30	1 g	tr	0	0	+	+	±	Strongly positive,	8+.
	A. I. D. H.	1:30	1 g	tr	0	0	+	+	±	Strongly positive,	8+.
	A. E. S. H.	1:30	1 g	+	+	tr	+	+	±	Weakly positive,	1+.
	A. I. S. H.	1:30	1 g	+	+	tr	+	+	±	Weakly positive,	1+.

TABLE 3—Continued
SIX DIFFERENT ANTIGENS COMPARED

Number of Serum	Kind of Antigen	Antigen Dilution	Amboceptor per Tube Unit	Readings						Results	
				Antigen Tubes			Control Tubes				
				1	2	3	1'	2'	3'		
77	A. E. H. H.	1:30	1 g	+	±	0	+	+	±	Strongly positive,	3+
	A. E. S. F. L.	1:40	1 g	+	0	0	+	+	±	Strongly positive,	5+
	A. E. D. H.	1:30	1 g	±	0	0	+	+	±	Strongly positive,	6+
	A. I. D. H.	1:30	1 g	+	+	tr	+	+	±	Weakly positive,	1+
	A. E. S. H.	1:30	1 g	+	+	0	+	+	±	Moderately positive,	2+
A. I. S. H.	1:30	1 g	+	+	±	+	+	±	Negative,	—	
78	A. E. H. H.	1:30	1 g	+	tr	0	+	+	±	Strongly positive,	4+
	A. E. S. F. L.	1:40	1 g	tr	0	0	+	+	±	Strongly positive,	5+
	A. E. D. H.	1:30	1 g	0	0	0	+	+	±	Strongly positive,	10+
	A. I. D. H.	1:30	1 g	+	+	tr	+	+	±	Weakly positive,	1+
	A. E. S. H.	1:30	1 g	+	tr	0	+	+	±	Strongly positive,	4+
A. I. S. H.	1:30	1 g	+	+	0	+	+	±	Moderately positive,	2+	
79	A. E. H. H.	1:30	1 g	tr	0	0	+	+	tr	Strongly positive,	6+
	A. E. S. F. L.	1:40	1 g	0	0	0	+	+	tr	Strongly positive,	8+
	A. E. D. H.	1:30	1 g	0	0	0	+	+	tr	Strongly positive,	8+
	A. I. D. H.	1:30	1 g	+	tr	0	+	+	tr	Strongly positive,	3+
	A. E. S. H.	1:30	1 g	tr	0	0	+	+	tr	Strongly positive,	6+
A. I. S. H.	1:30	1 g	+	±	0	+	+	tr	Moderately positive,	2+	
80	A. E. H. H.	1:30	1 g	+	+	0	+	+	±	Moderately positive,	2+
	A. E. S. F. L.	1:40	1 g	+	±	0	+	+	±	Strongly positive,	3+
	A. E. D. H.	1:30	1 g	+	±	0	+	+	±	Strongly positive,	3+
	A. I. D. H.	1:30	1 g	+	tr	0	+	+	±	Strongly positive,	4+
	A. E. S. H.	1:30	1 g	+	+	0	+	+	±	Moderately positive,	2+
A. I. S. H.	1:30	1 g	+	+	0	+	+	±	Moderately positive,	2+	
81	A. E. H. H.	1:30	1 g	+	+	tr	+	+	±	Weakly positive,	1
	A. E. S. F. L.	1:40	1 g	+	tr	0	+	+	±	Strongly positive,	4+
	A. E. D. H.	1:30	1 g	+	0	0	+	+	±	Strongly positive,	5+
	A. I. D. H.	1:30	1 g	+	+	±	+	+	±	Negative,	—
	A. E. S. H.	1:30	1 g	+	+	0	+	+	±	Moderately positive,	2+
A. I. S. H.	1:30	1 g	+	+	±	+	+	±	Negative,	—	
82	A. E. H. H.	1:30	1 g	+	+	tr	+	+	±	Weakly positive,	1+
	A. E. S. F. L.	1:40	1 g	+	+	0	+	+	±	Moderately positive,	2+
	A. E. D. H.	1:30	1 g	+	±	0	+	+	±	Strongly positive,	3+
	A. I. D. H.	1:30	1 g	+	±	±	+	+	±	Negative,	—
	A. E. S. H.	1:30	1 g	+	+	tr	+	+	±	Weakly positive,	1+
A. I. S. H.	1:30	1 g	+	+	±	+	+	±	Negative,	—	
83	A. E. H. H.	1:30	1 g	+	±	0	+	+	±	Strongly positive,	3+
	A. E. S. F. L.	1:40	1 g	+	0	0	+	+	±	Strongly positive,	5+
	A. E. D. H.	1:30	1 g	+	0	0	+	+	±	Strongly positive,	5+
	A. I. D. H.	1:30	1 g	+	+	tr	+	+	±	Weakly positive,	1+
	A. E. S. H.	1:30	1 g	+	±	0	+	+	±	Strongly positive,	3+
A. I. S. H.	1:30	1 g	+	+	tr	+	+	±	Weakly positive,	1+	
84	A. E. H. H.	1:30	1 g	+	+	tr	+	+	±	Weakly positive,	1+
	A. E. S. F. L.	1:40	1 g	+	tr	0	+	+	±	Strongly positive,	4+
	A. E. D. H.	1:30	1 g	+	±	0	+	+	±	Strongly positive,	3+
	A. I. D. H.	1:30	1 g	+	±	±	+	+	±	Negative,	—
	A. E. S. H.	1:30	1 g	+	+	0	+	+	±	Moderately positive,	2+
A. I. S. H.	1:30	1 g	+	+	±	+	+	±	Negative,	—	
85	A. E. H. H.	1:30	1 g	+	+	tr	+	+	±	Weakly positive,	1+
	A. E. S. F. L.	1:40	1 g	+	±	0	+	+	±	Strongly positive,	3+
	A. E. D. H.	1:30	1 g	+	±	0	+	+	±	Strongly positive,	3+
	A. I. D. H.	1:30	1 g	+	+	±	+	+	±	Negative,	—
	A. E. S. H.	1:30	1 g	+	+	tr	+	+	±	Weakly positive,	1+
A. I. S. H.	1:30	1 g	+	+	±	+	+	±	Negative,	—	

TABLE 3—Continued
SIX DIFFERENT ANTIGENS COMPARED

Number of Serum	Kind of Antigen	Antigen Dilution	Amboceptor per Tube Unit	Readings						Results
				Antigen Tubes			Control Tubes			
				1	2	3	1'	2'	3'	
86	A. E. H. H.	1:30	1 g	+	+	tr	+	+	±	Weakly positive, 1+. Strongly positive, 3+. Strongly positive, 3+. Negative, —. Weakly positive, 1+. Negative, —.
	A. E. S. F. L.	1:40	1 g	+	±	0	+	+	±	
	A. E. D. H.	1:30	1 g	+	±	0	+	+	±	
	A. I. D. H.	1:30	1 g	+	+	±	+	+	±	
	A. E. S. H.	1:30	1 g	+	+	tr	+	+	±	
	A. I. S. H.	1:30	1 g	+	+	±	+	+	±	
87	A. E. H. H.	1:30	1 g	+	+	0	+	+	±	Moderately positive, 2+. Strongly positive, 3+. Strongly positive, 5+. Weakly positive, 1+. Strongly positive, 3+. Negative, —.
	A. E. S. F. L.	1:40	1 g	+	±	0	+	+	±	
	A. E. D. H.	1:30	1 g	+	0	0	+	+	±	
	A. I. D. H.	1:30	1 g	+	+	tr	+	+	±	
	A. E. S. H.	1:30	1 g	+	±	0	+	+	±	
	A. I. S. H.	1:30	1 g	+	+	±	+	+	±	
88	A. E. H. H.	1:30	1 g	+	±	0	+	+	±	Strongly positive, 3+. Strongly positive, 5+. Strongly positive, 5+. Negative, —. Strongly positive, 3+. Weakly positive, 1+.
	A. E. S. F. L.	1:40	1 g	+	0	0	+	+	±	
	A. E. D. H.	1:30	1 g	+	0	0	+	+	±	
	A. I. D. H.	1:30	1 g	+	+	±	+	+	±	
	A. E. S. H.	1:30	1 g	+	±	0	+	+	±	
	A. I. S. H.	1:30	1 g	+	+	tr	+	+	±	
89	A. E. H. H.	1:30	1 g	+	0	0	+	+	±	Strongly positive, 5+. Strongly positive, 6+. Strongly positive, 6+. Moderately positive, 2+. Strongly positive, 5+. Weakly positive, 1+.
	A. E. S. F. L.	1:40	1 g	±	0	0	+	+	±	
	A. E. D. H.	1:30	1 g	±	0	0	+	+	±	
	A. I. D. H.	1:30	1 g	+	+	0	+	+	±	
	A. E. S. H.	1:30	1 g	+	0	0	+	+	±	
	A. I. S. H.	1:30	1 g	+	+	tr	+	+	±	
90	A. E. H. H.	1:30	1 g	±	0	0	+	+	±	Strongly positive, 6+. Strongly positive, 6+. Strongly positive, 6+. Weakly positive, 1+. Strongly positive, 6+. Negative, —.
	A. E. S. F. L.	1:40	1 g	±	0	0	+	+	±	
	A. E. D. H.	1:30	1 g	±	0	0	+	+	±	
	A. I. D. H.	1:30	1 g	+	+	tr	+	+	±	
	A. E. S. H.	1:30	1 g	±	0	0	+	+	±	
	A. I. S. H.	1:30	1 g	+	+	±	+	+	±	
91	A. E. H. H.	1:30	1 g	+	0	0	+	+	±	Strongly positive, 5+. Strongly positive, 6+. Strongly positive, 5+. Weakly positive, 1+. Strongly positive, 5+. Weakly positive, 1+.
	A. E. S. F. L.	1:40	1 g	±	0	0	+	+	±	
	A. E. D. H.	1:30	1 g	+	0	0	+	+	±	
	A. I. D. H.	1:30	1 g	+	+	tr	+	+	±	
	A. E. S. H.	1:30	1 g	+	0	0	+	+	±	
	A. I. S. H.	1:30	1 g	+	+	tr	+	+	±	
92	A. E. H. H.	1:30	1 g	+	+	0	+	+	±	Moderately positive, 2+. Moderately positive, 2+. Strongly positive, 3+. Negative, —. Moderately positive, 2+. Negative, —.
	A. E. S. F. L.	1:40	1 g	+	+	0	+	+	±	
	A. E. D. H.	1:30	1 g	+	±	0	+	+	±	
	A. I. D. H.	1:30	1 g	+	+	±	+	+	±	
	A. E. S. H.	1:30	1 g	+	+	0	+	+	±	
	A. I. S. H.	1:30	1 g	+	+	±	+	+	±	
93	A. E. H. H.	1:30	1 g	+	±	0	+	+	±	Strongly positive, 3+. Strongly positive, 4+. Strongly positive, 5+. Negative, —. Strongly positive, 4+. Negative, —.
	A. E. S. F. L.	1:40	1 g	+	tr	0	+	+	±	
	A. E. D. H.	1:30	1 g	+	0	0	+	+	±	
	A. I. D. H.	1:30	1 g	+	+	±	+	+	±	
	A. E. S. H.	1:30	1 g	+	tr	0	+	+	±	
	A. I. S. H.	1:30	1 g	+	+	±	+	+	±	
94	A. E. H. H.	1:30	1 g	+	0	0	+	+	±	Strongly positive, 5+. Strongly positive, 8+. Strongly positive, 8+. Weakly positive, 1+. Strongly positive, 8+. Negative, —.
	A. E. S. F. L.	1:40	1 g	tr	0	0	+	+	±	
	A. E. D. H.	1:30	1 g	tr	0	0	+	+	±	
	A. I. D. H.	1:30	1 g	+	+	tr	+	+	±	
	A. E. S. H.	1:30	1 g	tr	0	0	+	+	±	
	A. I. S. H.	1:30	1 g	+	+	±	+	+	±	

TABLE 3—Continued
SIX DIFFERENT ANTIGENS COMPARED

Number of Serum	Kind of Antigen	Antigen Dilution	Amboceptor per Tube Unit	Readings						Results	
				Antigen Tubes			Control Tubes				
				1	2	3	1'	2'	3'		
95	A. E. H. H.	1:30	1 g	+	±	0	+	+	±	Strongly positive,	3+.
	A. E. S. F. L.	1:40	1 g	+	0	0	+	+	±	Strongly positive,	5+.
	A. E. D. H.	1:30	1 g	±	0	0	+	+	±	Strongly positive,	6+.
	A. I. D. H.	1:30	1 g	+	+	tr	+	+	±	Weakly positive,	1+.
	A. E. S. H.	1:30	1 g	+	tr	0	+	+	±	Strongly positive,	4+.
	A. I. S. H.	1:30	1 g	+	+	tr	+	+	±	Weakly positive,	1+.
96	A. E. H. H.	1:30	1 g	+	+	tr	+	+	±	Weakly positive,	1+.
	A. E. S. F. L.	1:40	1 g	+	+	0	+	+	±	Moderately positive,	2+.
	A. E. D. H.	1:30	1 g	+	tr	0	+	+	±	Strongly positive,	4+.
	A. I. D. H.	1:30	1 g	+	+	±	+	+	±	Negative,	—.
	A. E. S. H.	1:30	1 g	+	+	tr	+	+	±	Weakly positive,	1+.
	A. I. S. H.	1:30	1 g	+	+	±	+	+	±	Negative,	—.
97	A. E. H. H.	1:30	1 g	+	±	0	+	+	±	Strongly positive,	3+.
	A. E. S. F. L.	1:40	1 g	+	tr	0	+	+	±	Strongly positive,	4+.
	A. E. D. H.	1:30	1 g	±	0	0	+	+	±	Strongly positive,	6+.
	A. I. D. H.	1:30	1 g	+	+	tr	+	+	±	Weakly positive,	1+.
	A. E. S. H.	1:30	1 g	+	±	0	+	+	±	Strongly positive,	3+.
	A. I. S. H.	1:30	1 g	+	+	±	+	+	±	Negative,	—.
98	A. E. H. H.	1:30	1 g	0	0	0	+	+	tr	Strongly positive,	8+.
	A. E. S. F. L.	1:40	1 g	0	0	0	+	+	tr	Strongly positive,	8+.
	A. E. D. H.	1:30	1 g	0	0	0	+	+	tr	Strongly positive,	8+.
	A. I. D. H.	1:30	1 g	+	0	0	+	+	tr	Strongly positive,	4+.
	A. E. S. H.	1:30	1 g	0	0	0	+	+	tr	Strongly positive,	8+.
	A. I. S. H.	1:30	1 g	+	tr	0	+	+	tr	Strongly positive,	3+.
99	A. E. H. H.	1:30	1 g	0	0	0	+	+	tr	Strongly positive,	8+.
	A. E. S. F. L.	1:40	1 g	0	0	0	+	+	tr	Strongly positive,	8+.
	A. E. D. H.	1:30	1 g	0	0	0	+	+	tr	Strongly positive,	8+.
	A. I. D. H.	1:30	1 g	+	±	0	+	+	tr	Moderately positive,	2+.
	A. E. S. H.	1:30	1 g	tr	0	0	+	+	tr	Strongly positive,	6+.
	A. I. S. H.	1:30	1 g	+	±	0	+	+	tr	Moderately positive,	2+.
100	A. E. H. H.	1:30	1 g	0	0	0	+	+	tr	Strongly positive,	8+.
	A. E. S. F. L.	1:40	1 g	tr	0	0	+	+	tr	Strongly positive,	6+.
	A. E. D. H.	1:30	1 g	0	0	0	+	+	tr	Strongly positive,	8+.
	A. I. D. H.	1:30	1 g	+	+	0	+	+	tr	Weakly positive,	1+.
	A. E. S. H.	1:30	1 g	+	0	0	+	+	tr	Strongly positive,	4+.
	A. I. S. H.	1:30	1 g	+	+	0	+	+	tr	Weakly positive,	1+.

insoluble antigen of dog heart usually gave weaker positive results than the alcoholic extract of human heart, but occasionally it gave a stronger positive result than any of the others. The results obtained with alcoholic extract of sheep heart were almost identical with those obtained with alcoholic extract of human heart, while the acetone insoluble antigen of sheep heart reacted somewhat weaker.

TEST 4

In order to determine whether glycerol in any way influenced the result of the Wassermann reaction with dog antigen, 10 serums were tested before and after having been glycerolated. Each serum was divided into 2 portions, A and B, and both portions were heated to about 56 C. for 30 minutes.

Portion A was diluted with an equal volume of salt solution, Portion B was mixed with an equal volume of glycerol, and both portions were tested against alcoholic extract of human heart and against alcoholic extract of dog heart.

TABLE 4
NONGLYCEROLATED AND GLYCEROLATED SERUM COMPARED

Number of Serum	Portions A=Non- glycero- lated B=Glycer- olated	Kind of Antigen	Ambo- ceptor per Tube Unit	Readings						Results	
				Antigen Tubes			Control Tubes				
				1	2	3	1'	2'	3'		
101	A	A. E. H. H.	1	+	+	tr	+	+	tr	Negative,	—
	A	A. E. D. H.	1	+	+	tr	+	+	tr	Negative,	—
	B	A. E. H. H.	1 g	+	+	±	+	+	±	Negative,	—
	B	A. E. D. H.	1 g	+	+	±	+	+	±	Negative,	—
102	A	A. E. H. H.	1	+	+	±	+	+	±	Negative,	—
	A	A. E. D. H.	1	+	+	±	+	+	±	Negative,	—
	B	A. E. H. H.	1 g	+	+	±	+	+	±	Negative,	—
	B	A. E. D. H.	1 g	+	+	±	+	+	±	Negative,	—
103	A	A. E. H. H.	1	+	+	±	+	+	±	Negative,	—
	A	A. E. D. H.	1	+	+	±	+	+	±	Negative,	—
	B	A. E. H. H.	1 g	+	+	±	+	+	±	Negative,	—
	B	A. E. D. H.	1 g	+	+	±	+	+	±	Negative,	—
104	A	A. E. H. H.	1	+	+	±	+	+	±	Negative,	—
	A	A. E. D. H.	1	+	+	±	+	+	±	Negative,	—
	B	A. E. H. H.	1 g	+	+	±	+	+	±	Negative,	—
	B	A. E. D. H.	1 g	+	+	±	+	+	±	Negative,	—
105	A	A. E. H. H.	1	+	+	±	+	+	±	Negative,	—
	A	A. E. D. H.	1	+	+	±	+	+	±	Negative,	—
	B	A. E. H. H.	1 g	+	+	±	+	+	±	Negative,	—
	B	A. E. D. H.	1 g	+	+	±	+	+	±	Negative,	—
106	A	A. E. H. H.	1	+	+	±	+	+	±	Negative,	—
	A	A. E. D. H.	1	+	+	tr	+	+	±	Weakly positive,	1+
	B	A. E. H. H.	1 g	+	+	±	+	+	±	Negative,	—
	B	A. E. D. H.	1 g	+	±	0	+	+	±	Strongly positive,	3+
107	A	A. E. H. H.	1	+	+	±	+	+	±	Negative,	—
	A	A. E. D. H.	1	+	±	0	+	+	±	Strongly positive,	3+
	B	A. E. H. H.	1 g	+	+	±	+	+	±	Negative,	—
	B	A. E. D. H.	1 g	+	0	0	+	+	±	Strongly positive,	5+
108	A	A. E. H. H.	1	+	+	±	+	+	±	Negative,	—
	A	A. E. D. H.	1	+	+	tr	+	+	±	Weakly positive,	1+
	B	A. E. H. H.	1 g	+	+	±	+	+	±	Negative,	—
	B	A. E. D. H.	1 g	+	±	0	+	+	±	Strongly positive,	3+
109	A	A. E. H. H.	1	+	+	±	+	+	±	Negative,	—
	A	A. E. D. H.	1	+	+	tr	+	+	±	Weakly positive,	1+
	B	A. E. H. H.	1 g	+	+	±	+	+	±	Negative,	—
	B	A. E. D. H.	1 g	+	±	0	+	+	±	Strongly positive,	3+
110	A	A. E. H. H.	1	+	+	±	+	+	±	Negative,	—
	A	A. E. D. H.	1	+	+	±?	+	+	±	Faintly positive,	±
	B	A. E. H. H.	1 g	+	+	±	+	+	±	Negative,	—
	B	A. E. D. H.	1 g	+	±	0	+	+	±	Strongly positive,	3+

Table 4 shows that all serums which gave positive results with alcoholic extract of dog heart after they had been mixed with glycerol also gave positive results before they had been mixed with glycerol.

The glycerolated portions gave stronger positive results than did the nonglycerolated portions, but this is to be expected with any antigen. Glycerol makes the test more sensitive.

TEST 5

Eight serums were tested against alcoholic extract of dog heart before and after their natural antidog amboceptor had been absorbed. Each serum was divided into two portions, A and B. Portion A was left whole while from Portion B the antidog amboceptor was absorbed. Both portions were tested against alcoholic extract of dog heart.

TABLE 5
HUMAN SERUM TESTED BEFORE AND AFTER ABSORPTION OF ANTIDOG AMBOCEPTOR

Number of Serum	Portions A, Amboceptor Not Absorbed B, Amboceptor Absorbed	Kind of Antigen	Amboceptor per Tube Unit	Readings						Results
				Antigen Tubes			Control Tubes			
				1	2	3	1'	2'	3'	
111	A	A. E. D. H.	1 g	+	±	0	+	+	±	Strongly positive, 3+.
	B	A. E. D. H.	1 g	+	±	0	+	+	±	Strongly positive, 3+.
112	A	A. E. D. H.	1 g	+	+	0	+	+	±	Moderately positive, 2+.
	B	A. E. D. H.	1 g	+	+	0	+	+	±	Moderately positive, 2+.
113	A	A. E. D. H.	1 g	+	±	0	+	+	±	Strongly positive, 3+.
	B	A. E. D. H.	1 g	+	±	0	+	+	±	Strongly positive, 3+.
114	A	A. E. D. H.	1 g	+	±	0	+	+	±	Strongly positive, 3+.
	B	A. E. D. H.	1 g	+	±	0	+	+	±	Strongly positive, 3+.
115	A	A. E. D. H.	1 g	+	0	0	+	+	±	Strongly positive, 5+.
	B	A. E. D. H.	1 g	+	0	0	+	+	±	Strongly positive, 5+.
116	A	A. E. D. H.	1 g	±	0	0	+	+	±	Strongly positive, 6+.
	B	A. E. D. H.	1 g	±	0	0	+	+	±	Strongly positive, 6+.
117	A	A. E. D. H.	1 g	+	±	0	+	+	±	Strongly positive, 3+.
	B	A. E. D. H.	1 g	+	±	0	+	+	±	Strongly positive, 3+.
118	A	A. E. D. H.	1 g	+	tr	0	+	+	±	Strongly positive, 4+.
	B	A. E. D. H.	1 g	+	tr	0	+	+	±	Strongly positive, 4+.

The results are shown in Table 5. Absorbing the natural antidog amboceptor from human serum did not affect the results obtained with the Wassermann test using alcoholic extract of dog heart as antigen.

SUMMARY

Antigen diluted with the salt solution slowly so as to give an opalescent solution gave many more positive results with the Wassermann test than did antigen which was diluted rapidly enough to give a clear solution.

Adding the antigen to the human serum before the complement gave results identical with those obtained when the complement was added before the antigen.

Antigen supposed to be alcoholic extract of syphilitic fetal liver and antigen prepared from dog heart gave many more positive results than did alcoholic extract of human heart. As no other signs or symptoms of syphilis could be detected these were considered false positive results, and these antigens were discarded as being unreliable. Antigen prepared from sheep heart gave results almost identical with those given by antigen prepared from human heart.

The glycerol added to the human serum cannot be held responsible for the false positive results obtained.

Previous absorption of the natural antidog amboceptor from the human serums did not eliminate the false positive results.

TRANSMISSIBILITY OF IMMUNITY FROM MOTHER TO OFFSPRING IN HOG CHOLERA

CLIFFORD L. MCARTHUR

From the Department of Bacteriology and Pathology, Arkansas Experiment Station, Fayetteville, Ark.

In earlier publications different investigators have noted that pigs from immune mothers possess a certain amount of immunity to hog cholera. The extent of this immunity as well as its duration varies somewhat in the different reports. While several reports are noted from observations made in the field and not under experimental conditions, the following review of the literature gives a brief summary of the experimental data which is available on the subject.

Peters¹ found that hogs recovering from an attack of hog cholera were immune, as were also their litters. Pigs farrowed from sows which were vaccinated with the double treatment 3 weeks previous to farrowing, were not immune after 5 months. Jacob² reports that sows treated with antiserum did not become infected with hog cholera while their litters died with the disease. He does not state, however, whether or not the pigs were suckling when they became infected. The report by Reynolds³ states that litters farrowed from immune sows are highly immune to the disease. He reports further that if these pigs are given small amounts of virus they will withstand the injection and become actively immune, otherwise the immunity is eventually lost after weaning. The sows used in his work were immune from passing through an outbreak of the disease and not from vaccination.

In the experimental work conducted thus far by different investigators, the greater part of the work has not been conducted under control conditions, as most of the sows used in the experiments were immune from having passed through an outbreak of the disease, and but few were rendered immune by the Dorset-Niles method of treatment.

Because of the importance of this question and the small amount of experimental data available, the following experiments were conducted with sows immune from the Dorset-Niles treatment, in order that further information might be secured. The fact that only swine are susceptible to hog cholera besets the study of this transmitted immunity

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¹ Neb. Sta. Report, 1909, p. 111.

² Tenn. Sta. Report, 1909, p. 112.

³ Am. Vet. Rev., 1910, 38, p. 236.

with more or less difficulty. In order to study the subject thoroughly one should be able to secure milk from the mother as well as blood samples from both mother and offspring and test these for immune bodies. In the case of hogs, milk can rarely be secured, and then only with difficulty; further, there is as yet no laboratory test which can be applied for this disease.

All sows used in these experiments were secured from the surrounding country. These sows had never been treated with antihog cholera serum, and so far as known were not immune from exposure or passing through an outbreak of the disease. The sows varied in weight from 200-350 lbs., and whenever possible, sows were secured which had previously farrowed 1 or 2 litters. As soon as these sows were delivered to the experimental pens they were given the Dorset-Niles antihog cholera serum with virus. The quantity of serum and virus used being that recommended in the regular treatment according to the weight of the hog. In a few instances the serum alone without the virus was given, as will be noted under the different experiments. Practically all of the sows were pregnant when vaccinated. After treatment the sows were placed in sheds 8 by 13 feet with concrete floor. They remained in these sheds until after the litters were weaned.

Exper 1.—Two classes of sows were included in this experiment. Those receiving antiserum without virus and those receiving both antiserum and virus but showing only a passive immunity after treatment. As shown in the following table some of the litters were exposed to virus pigs when 4 days old. All pigs which survived were given an injection of virus after weaning to test the extent of their immunity. Data was secured from 5 litters.

Sow No.	Number of Pigs in Litter	First Treatment	Second Treatment	Number of Pigs Showing Cholera Lesions on Necropsy	Number of Pigs Without Cholera Lesions on Necropsy	Number of Pigs Surviving
1	3	Exposed to virus pigs when 4 days old	Sow sickened. Given 180 cc of antihog cholera serum	3	0	0
3	4	Exposed to virus pigs when 4 days old	Surviving pigs given 0.5 cc of virus 6 weeks after weaning	0	1	3
8	5	0	5	0
	All dead; sow died					
10	4	Exposed to virus pigs when 4 days old	Sow died of cholera before pigs were weaned	2	2	0
16	5	No treatment	Sow and pigs died 2 days after farrowing; sow with cholera	0	5	0

The results of the foregoing experiment would indicate that the pigs were immune as long as the mother was immune and the pigs were suckling. As soon as the sow sickened practically all of the pigs contracted the disease and died. All of the surviving pigs of sow No. 3 were still immune 6 weeks after weaning and withstood an injection of virus.

Exper 2.—Only sows, which had received both antihog cholera serum and virus and were proven immune, were used in this experiment. The litters as well as the sows were exposed to hog cholera by placing in the pen virus pigs with well marked cases of the disease. The virus pigs were sometimes allowed to remain in the pens from 12-24 hours after death.

Sow No.	Number of Pigs in Litter	First Treatment	Second Treatment	Number of Pigs Showing Cholera Lesions on Necropsy	Number of Pigs Without Cholera Lesions on Necropsy	Number of Pigs Surviving
2	6	Exposed to virus pig when 2 days old	Exposed to virus pig after weaning	0	3	3
4	4	Exposed to virus pig when 1 week old	Exposed to virus pig after weaning	0	1	3
6	8	Exposed to virus pig when 1 day old	Exposed to virus pig after weaning	0	1	7
9	6	Exposed to virus pig when 1 day old	Exposed to virus pig after weaning	1	1	4
14	5	Exposed to virus pig when 1 day old	Exposed to virus pig after weaning	1	1	3

Of the different pigs which died in this experiment, only 2 showed lesions of hog cholera. All of the surviving pigs were exposed to virus pigs twice, once while suckling and once after weaning. Three of the pigs were given an injection of 0.25 cc of virus over 2 months after weaning and still survived. From the large percentage which survived it is evident that these pigs were highly immune.

Exper. 3.—The sows in this experiment had all received antihog cholera serum and virus and were proven actively immune after treatment. The experiment differs from the previous experiments, in that the pigs received

Sow No.	Number of Pigs in Litter	First Treatment	Second Treatment	Number of Pigs Showing Cholera Lesions on Necropsy	Number of Pigs Without Cholera Lesions on Necropsy	Number of Pigs Surviving
5	5	Teats of sow smeared with virus	Exposed to virus pig after weaning	0	2	3
12	3	Teats of sow smeared with virus	Exposed to virus pig after weaning	0	0	3
25	9	Teats of sow smeared with virus	Exposed to virus pig after weaning	1	1	7
29	7	Teats of sow smeared with virus	Exposed to virus pig after weaning	0	1	6
30	4	Teats of sow smeared with virus	Exposed to virus pig after weaning	1	0	3

virus by way of the stomach and digestive tract. In each case 2 days after the pigs were farrowed the teats of the mother were smeared with virulent hog cholera virus. In this way each pig received some of the virus while suckling. All surviving pigs were exposed to virus pigs to determine whether or not they were immune after weaning.

Of the entire number of pigs which died in this experiment only 2 showed cholera lesions. A large percentage of each litter survived the treatment. This would indicate that these pigs were highly immune to hog cholera infection by way of the digestive tract so long as they were suckling.

Exper. 4.—As in the previous experiment, the sows in this experiment received both antiserum and virus and were immune to the disease. The experiment differs from the previous experiments in that each pig was given an injection of virus while suckling as noted in the following table. All surviving pigs were given another injection of virus after weaning.

Sow No.	Number of Pigs in Litter	First Treatment	Second Treatment	Number of Pigs Showing Cholera Lesions on Necropsy	Number of Pigs Without Cholera Lesions on Necropsy	Number of Pigs Surviving
7	9	Given 0.5 c c of virus when 1 week old	Given 1 c c of virus 2 weeks after weaning	2	6	1
11	6	Given 0.5 c c of virus when 4 weeks old	Given 1 c c of virus 5 days after weaning	0	2	4
15	8	Given 0.5 c c of virus when 1 week old	Given 1 c c of virus 2 weeks after weaning	1	1	6
19	7	Given 0.5 c c of virus when 2 weeks old	Given 1 c c of virus 2 weeks after weaning	0	0	7
26	7	Given 0.5 c c of virus when 1 week old	Given 1 c c of virus 2 weeks after weaning	0	1	6

A large percentage of the pigs withstood the injection of virus both before and after weaning. While the death rate was rather high, only 3 pigs showed hog cholera lesions on necropsy. The pigs were as highly immune to the injections of virus as to any of the methods of exposure in the previous experiments.

Exper. 5.—All of the sows were actively immune to hog cholera from treatment with antiserum and virus. This experiment differs from all of the preceding experiments in that the litters were not exposed to hog cholera until after weaning. Each pig was given an injection of virus from 2 days to 2 weeks after weaning. This was to determine whether or not their immunity was lost soon after weaning.

Sow No.	Number of Pigs in Litter	Treatment	Number of Pigs Showing Cholera Lesions on Necropsy	Number of Pigs Without Cholera Lesions on Necropsy	Number of Pigs Surviving
13	1	Given 1 c c of virus 2 days after weaning	1	0	0
17	5	Given 0.5 c c of virus 2 weeks after weaning	0	0	5
20	7	Given 0.5 c c of virus 4 days after weaning	0	1	6
21	5	Given 0.5 c c of virus 1 week after weaning	2	0	3
22	5	Given 0.5 c c of virus 2 days after weaning	0	0	5
23	9	Given 0.5 c c of virus 4 days after weaning	0	0	9

In nearly every case these pigs survived the injection of virus when given from 2 days to 2 weeks after weaning. These results would indicate that the litters were as highly immune shortly after weaning as before weaning.

Exper. 6.—The data in this experiment were from the second litter farrowed since the mother was vaccinated. In this way it was hoped to determine the degree of immunity of the second litter as compared to the first litter from the same sow. The sows were all immune to hog cholera having received both antiserum and virus. In practically all cases the sows were vaccinated at least 6 months before these litters were farrowed. The litters were not exposed to hog cholera until after weaning, when they were given an injection of virus.

Sow No.	Number of Pigs in Litter	Treatment	Number of Pigs Showing Cholera Lesions on Necropsy	Number of Pigs Without Cholera Lesions on Necropsy	Number of Pigs Surviving
3	6	Given 0.5 c c of virus 2 days after weaning	0	0	6
5	9	Given 0.5 c c of virus 4 days after weaning	0	2	7
17	5	Given 0.5 c c of virus 2 weeks after weaning	0	0	5
18	8	Given 0.5 c c of virus 1 week after weaning	0	0	8
21	4	Given 0.5 c c of virus 2 days after weaning	0	2	2

These results would indicate that second litters are possibly more highly immune than first litters, as the death rate was low and none of the pigs showed cholera lesions. It is evident that second litters are immune to hog cholera so long as they are suckling an immune mother.

SUMMARY

The results of these experiments would indicate that sows immunized against hog cholera by the Dorset-Niles method transmit this immunity to their offspring. In nearly every case the pigs retained

their immunity as long as they were suckling and the sow was immune. When the mother contracted the disease the pigs did not usually survive more than a few days, and in some instances cholera lesions were found on necropsy.

There seemed to be but little difference in susceptibility of these suckling pigs to different methods of exposure.

The duration of immunity in the different pigs, after weaning, was only tested to a limited extent. From the data available, it is evident that different pigs vary, but in most cases the immunity lasted a few weeks after weaning.

A number of pigs died in the different experiments without showing cholera lesions. Death in these cases was probably due to indigestion, worms, etc., and not to hog cholera.

Second litters were as highly immune as the first litters in all cases, and in some instances even more highly immune while suckling.

The method whereby the antibodies are transmitted from mother to offspring in hog cholera is more or less an open question. The fact that the suckling litters sicken as soon as the mother becomes infected, also, that immunity is gradually lost after weaning, would indicate that the antibodies are transmitted through the milk during the entire suckling period.

Data were secured from 31 litters, covering a total of 179 pigs. Of this number 125, or 69.8%, survived all treatment; 39, or 21.7%, succumbed during the course of the experiments, but did not show cholera lesions on necropsy and 15 pigs, or 8.3%, died showing cholera lesions. From these results it might be considered that 91.7% of the pigs were immune to hog cholera. It is entirely possible, however, that some of the pigs which died without cholera lesions were affected more or less with the disease, and that the percentage of pigs showing immunity would be less than 91.7%.

THE RESISTANCE OF THE GLANDERS BACILLUS TO CALCIUM HYPOCHLORITE

BARNETT COHEN

Department of Public Health, Yale University School of Medicine, New Haven, Conn.

A brief survey of available literature reveals a lack of definite information as to the resistance of the glanders bacillus to calcium hypochlorite (chlorid of lime), though there is a consensus of opinion among writers of textbooks on bacteriology that this organism is highly parasitic and quite sensitive to external physical and chemical agencies. For instance, a temperature below 22 C. is unfavorable for growth; and exposure to 75 C. for 1 hour, to 5% carbolic acid for $\frac{1}{2}$ hour, or to 1:1,000 bichlorid of mercury for 15 minutes will destroy the organism.

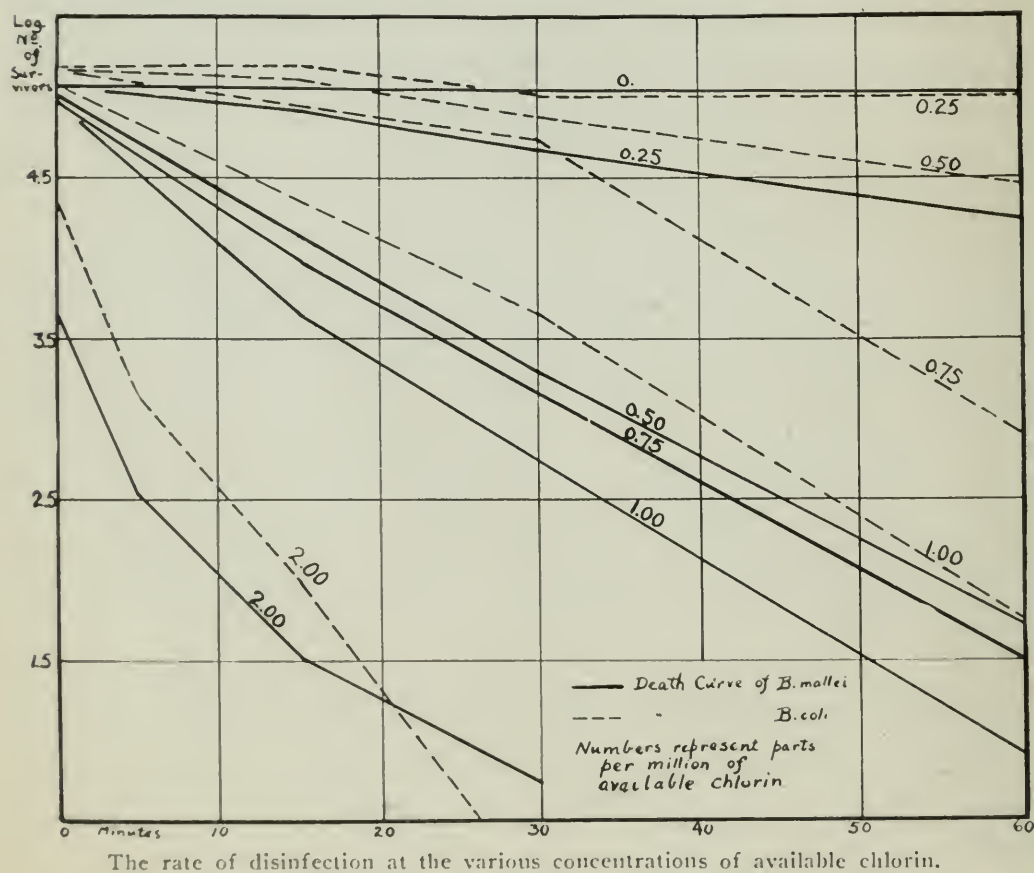
The question of the longevity in water of the glanders bacillus has attracted attention because of its relation to the infection of horses through use of drinking troughs. In this regard it appears, according to Hiss and Zinsser, that the glanders bacillus may remain alive for 70 days in the water of horse troughs. It is therefore within the realm of possibility that an infection of a municipal water supply with this organism might be accomplished, whether by accident or intention. In view of such a possibility, however remote, it seemed desirable to determine the resistance of the glanders bacillus to hypochlorite in the strengths customarily employed for the disinfection of water supplies of cities.

The plan followed was to expose a suspension of the organism to concentrations of available chlorin of 0.10, 0.25, 0.50, 0.75, 1.00, and 2.00 parts per million, respectively, and determine at successive intervals the numbers of the survivors. From these data, curves may be constructed showing the corresponding rates of decline in numbers.

The culture was grown on a glycerol agar slant for 18 hours at 37.5 C.; and the growth was then washed off with 0.95% salt solution, the suspension being thoroughly shaken, and suitably diluted for the required tests. Of the hypochlorite, 0.3 gm. was dissolved in distilled water and filtered. To determine the amount of available chlorin it contained, this stock solution was titrated for each test with standard N/10 sodium thiosulfate. The calculated amount of stock hypochlorite solution was then accurately added to 100 c.c. of sterile tap water to give to it the required concentration of available chlorin. (Tap water was used to approximate natural conditions. One series

of tests in which distilled water was used, yielded practically the same results as those here reported.)

A small amount (0.1-1 cc) of the bacterial suspension was added to this last mentioned solution, the bottle quickly and thoroughly shaken, and 1 cc of the contents plated in suitable dilutions to determine the initial number of organisms present. The plating medium was 1% glycerol agar with a P_H of 6.6-7.0, and incubation of the plates was for 4 days at 37.5 C. The bottles were kept in diffuse light at room temperature (about 22 C.) which did not vary more than 2 or 3 degrees throughout the experiment; and at determined intervals up to 1 hour, samples were removed to determine the corresponding number of survivors. It was assumed that 1 hour was the maximum period of exposure that would normally occur in practice.



Briefly, then, the procedure in these experiments was to (1) make the bacterial suspension; (2) titrate the stock hypochlorite solution; (3) prepare solution with known concentration of available chlorin; (4) add a little of the bacterial suspension to this solution of known chlorin content; (5) remove a 1 cc sample at suitable intervals and plate; (6) count the colonies on the plates after 4 days' incubation at 37.5 C.

It became evident early in these experiments that, to judge the resistance of the glanders bacillus properly, it was necessary to use another organism for comparison. *Bacillus coli* was chosen because of its known sensitiveness to calcium hypochlorite; and parallel tests were conducted on both *B. mallei* and *B. coli*.

Under the conditions of our experiments, there was only a slight or no diminution in numbers when the organisms were exposed to a concentration of $\frac{1}{10}$ part per million of available chlorin. This is probably accounted for by removal of the available chlorin by the relatively large amount of organic matter necessarily transferred in the bacterial suspension.

These results are not, therefore, directly comparable with those obtained in the disinfection of water supplies where (in the absence of much organic matter) small concentrations of chlorin are often effective. It is the comparative resistance of the two organisms which is significant. It will also be noted that concentrations of 0.5 or more parts per million of available chlorin are greater than normally used in the disinfection of water supplies. These were, however, included in this work in order to indicate the possible efficiency of hypochlorite in the disinfection of watering troughs.

TABLE 1
SURVIVORS PER C C OF *B. MALLEI* AND *B. COLI* AFTER EXPOSURE TO DIFFERENT
CONCENTRATIONS OF 'AVAILABLE' CHLORIN FOR DIFFERENT PERIODS

Available Chlorin, Parts per Million	Time				
	0	5 Minutes	15 Minutes	30 Minutes	60 Minutes
<i>B. Mallei</i>					
0	111,000	121,000	110,000	103,000
0.25	108,000	84,000	47,000	16,500
0.50	101,000	12,100	1,980	50
0.75	93,000	11,500	1,640	29
1.00	95,000	4,700	770	8
2.00	4,100	340	33	11	—
<i>B. Coli</i>					
0	138,000	97,400	
0.25	142,000	155,000	104,000	101,000
0.50	153,000	127,000	74,000	29,000
0.75	139,000	87,000	53,000	800
1.00	114,000	4,600	55
2.00	21,000	1,400	100	0	—

Table 1 shows the results of a typical experiment in which the initial numbers of organisms were comparatively low (about 150,000 per c c). Another series of experiments not reported here in which large initial numbers (15 million per c c) of organisms were employed,

gave substantially the same results, except the larger amount of organic matter caused a somewhat slower disinfection rate.

The accompanying chart gives a clear picture of the rate of disinfection at the various concentrations of available chlorin. The abscissae represent time in minutes, and the ordinates the logarithms of the numbers of survivors. Thus the slope of any curve in this chart shows the actual rate of decrease — the steeper the curve downward, the greater the rate of decrease.

The actual sensitivity of *B. mallei* to hypochlorite in a water supply, as pointed out in the foregoing, cannot be accurately judged from the death curves of this organism considered alone. The conditions of our experiments were such as to diminish greatly the effect of low concentrations of available chlorin; and even at the higher concentrations, one might be led to conclude erroneously that the glanders bacillus was quite resistant. When, however, the corresponding curves for *B. mallei* and *B. coli* are compared, it is seen that, if anything, *B. mallei* is more sensitive to hypochlorite than *B. coli*. It is well known that *B. coli* in water supplies is quite sensitive to hypochlorite in very low concentration, over 90% reduction being obtained in very short time. Therefore, we may conclude that under normal conditions of water-works practice in our larger cities, where the drinking water is continually chlorinated, *B. mallei* will have little chance to survive longer than *B. coli*.

It will also be noticed in the results here reported that practical sterility is gained in about 30 minutes with an exposure to 2 parts per million of available chlorin. This, however, applies only to a condition in which there is a rather light seeding of organisms and absence of considerable organic matter. Our evidence indicates, as would be expected, a diminution of disinfecting power of hypochlorite toward *B. mallei* in the presence of organic matter. This should be remembered when considering the question of the disinfection of horse troughs. In spite of this drawback, we have found that over-night exposure of *B. mallei* to 2 parts per million of available chlorin, even in the presence of considerable organic matter such as leaves and stems, has killed it, or at least effectively removed its power to grow when transferred to a favorable medium. One might reasonably conclude from this that sterilization of horse troughs could be easily effected with hypochlorite in relatively small amounts. Hypochlorite could be employed as a prophylactic measure in the water of horse troughs where glanders infection is prevalent.

SUMMARY

Studies were made on the specific resistance of the glanders bacillus toward calcium hypochlorite in concentrations employed for the disinfection of water supplies.

When compared to *B. coli*, *B. mallei* is, if anything, more sensitive toward hypochlorite.

Hypochlorite may be effectively used in the disinfection of horse troughs as a harmless prophylactic measure in glanders infected regions.

SPIROCHETE-LIKE SPIRAL BODIES IN BACTERIAL CULTURES

PLATE 1

G. KOGA AND G. OTSUBO

From the Kitasato Institute for Infectious Diseases, Tokyo

While attempting to obtain pure culture of smegma spirochetes, we encountered not a little difficulty which consisted in the appearance of a large number of spiral bodies resembling spirochetes. They can be detected either under the dark field microscope or in the india ink method in the special medium after 15 hours' incubation. These bodies have a striking resemblance to the involuted form of *Treponema macrodentium* which Noguchi¹ found in the buccal cavity. According to this investigator, they reach their maximum growth on the 2nd or 3rd day, and afterward gradually decrease in number, the process being much quicker in the presence of other bacteria producing gas and acids. Sometimes this form does not appear at all. Because of such precarious nature, its pure culture is a most difficult task. However, once started, it is comparatively easy to transplant it.

To obtain pure culture of the spiral bodies in question, we tried in vain both the filtration method and Noguchi's purification. We also tried anaerobic plate isolation in hydrogen, using plasma-ascites medium, Noguchi's ascites-fluid agar, and Shimamine's horse serum medium, incubating at 22 C. for from 3-7 days. On examination we could not find any pure colony of the spiral bodies. We could, however, detect them mixed with *Bacillus subtilis*. Thus, we only succeeded in obtaining the mixed cultures of these two kinds of organisms. All the possible methods we tried failed to separate them, leading us to the conclusion that there might be some symbiotic relation between them. We had, therefore, to study the morphology of the spiral bodies under consideration in the mixed culture.

Form.—From the results of the examination of several strains of the spiral bodies under the dark field microscope or with the india ink

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¹ Jour. Exper. Med., 1912, 15, p. 81

method, we can distinguish two distinct forms: (a) large spirochete-like bodies, as we may provisionally call them, have a rather small number of spirals, each 2-5 mikrons in length and 1-1.5 mikrons in width; (b) small spirochete-like bodies with short spirals (1-1.5 mikrons in length by 1-1.2 mikrons in width), at nearly a right angle with one another. In both, the forms of the spirals are regular. The number of spirals and the length varies a great deal; the former from two to several hundred spirals, the latter four to several hundred spirals. Usually, however, they have 6-15 spirals and a length of 15-30 mikrons. They are mostly straight. Sometimes they show various forms of curvatures and sometimes they assume the shape of entangled threads or bundles. Often they give an appearance of a longitudinal split, as described by Noguchi. However, transverse divisions are not lacking.

Motility.—We have never come across any motile form.

Stainability.—The spiral bodies do not have any affinity toward ordinary anilin dyes or strong staining fluids such as carbolfuchsin, anilin, gentian violet, caustic potash-methylene blue. However, since the other portions are stained with these fluids, the spiral bodies can be detected as negatives, so to speak. They refuse to take either Giemsa's fluid or its substitute—Otani's azur-eosin solution. They can, on the contrary, be beautifully stained by Löffler's flagellum staining method and by Zettnow's² silver impregnation.

Resistance Against Temperature.—The mixed cultures of *Bacillus subtilis* and the spiral bodies under consideration were mixed with the salt solution, and exposed to various degrees of temperature during various lengths of time and then inoculated in the plasma-ascites medium. They die in 1 hour at 85 C., in half an hour at 90 C., while at 60 C. the development was checked only after heating for 1 hour for 3 days in succession. *B. subtilis* accompanying the spiral bodies was found to have the same fate as the latter. The result may be tabulated as follows:

TABLE 1
EFFECT OF TEMPERATURE ON THE SPIRAL BODIES AND BACTERIAL CULTURES

	60 C.	65 C.	70 C.	80 C.	85 C.	90 C.
25 minutes.....	+	+	+	+	+	+
30 minutes.....	+	+	+	+	+	—
One hour.....	+	+	+	+	—	—

² Deutsch. med. Wchnschr., 1906, 32, p. 376.

The microscopic examination of the spiral bodies in the heating apparatus revealed that they considerably decrease in number after 10 minutes at 60 C. and 5 minutes at 70 C., and that they are completely disorganized after 15 minutes at 70 C. Yet when the debris was transplanted into the proper medium, a large number of the spiral bodies was seen to develop.

TABLE 2
EFFECT OF DIFFERENT TEMPERATURES ON SPIRAL BODIES

Temperature	Before Heating	5 Minutes	10 m.	15 m.	20 m.	30 m.	40 m.	50 m.	60 m.
60° C.....	+++	+++	+++	++	+	+	+	+	+
70° C.....	+++	++	+	—	—	—	—	—	—

Resistance Against Disinfectants.—The spiral bodies are disorganized immediately by 5% formalin, 5% carbolic acid and 90% alcohol, but 0.1-0.5% corrosive sublimate, 0.1% potassium permanganate, and 1% antiformin, do not disorganize them. But in all of these cases a multitude of the spiral bodies were seen to develop in the ascites-plasma medium into which thus treated material was inoculated; 5% antiformin, however, was found strong enough to kill them immediately.

TABLE 3
EFFECT OF DISINFECTANTS ON SPIRAL BODIES

Disinfectants	Before Mixing	Imme- diately After Mixing	5 Min- utes	10 m.	15 m.	20 m.	25 m.	30 m.	Reinoc- ulation After 30 min.
5% formalin.....	+	—	—	—	—	—	—	—	+
5% carbolic acid.....	+	—	—	—	—	—	—	—	+
90% alcohol.....	+	—	—	—	—	—	—	—	+
0.1% sublimate.....	+	+	+	+	+	+	+	+	+
0.5% sublimate.....	+	+	+	+	+	+	+	+	+
0.1% potassium permanganate	+	+	+	+	+	+	+	+	+
1% antiformin.....	+	+	+	+	+	+	+	+	+
5% antiformin.....	+	—	—	—	—	—	—	—	—

Cultivation.—We have found that the spiral bodies grow best in our plasma-ascites medium. They also grow on Noguchi's ascites agar, and Shimamine's horse serum medium. They do not develop at all in medium lacking fresh proteins.

Parenthetically we would describe the method of preparing our plasma-ascites medium: Into the horse blood serum citrate is added in proportion of 0.4% and the citrated blood is kept in the refrigerator, until the corpuscles are settled entirely. The plasma thus obtained is mixed with the same quantity of the ascites fluid (sp. gr. 1.010). A 10% solution of calcium chlorid is added to the mixture in proportion of 1 cc to 40 cc. The medium thus prepared is poured into the test tubes.

Nature of the Spiral Bodies.—From what has been described, it may be concluded that the spiral bodies correspond morphologically to spirochetes, yet they are differentiated from the latter in the following features: (*a*) lack of motion; (*b*) difference in the stainability; (*c*) powerful resistance to heat and disinfectants; (*d*) quick growth as compared with that of the genuine spirochetes, and lastly (*e*) the absolute impossibility to isolate them. Of these, the last characteristic is the most interesting and it may be worth while to describe it in more detail.

Since we failed to separate the spiral bodies from *Bacillus subtilis*, we reversed the process, that is, we tried to isolate *B. subtilis* from the spiral bodies, the culture of *B. subtilis* being made on the plating agar medium. The colonies were proved to be pure by microscopic examination. After their purity had been ascertained, they were inoculated in the plasma-ascites medium to see if the problematical bodies might appear. We found that they did in great number, intermingled with the *subtilis* bacilli. We transplanted the bacilli grown in plasma-ascites to the plain agar medium. We cultivated the *subtilis* for more than 20 generations on the agar. Every time the reinoculation was made, the germs were also inoculated into the plasma-ascites medium, and each time they were observed to be mixed with the spiral bodies, while on the agar medium *B. subtilis* alone grew.

TABLE 4
SHOWING THAT A SPECIAL RELATION EXISTS BETWEEN SPIRAL BODIES AND *B. SUBTILIS*

	Generation 1	Generation 2	Generation 3	Generation 4
Medium.....	Agar	Agar	Agar	Agar
Spiral body.....	—	—	—	—
Medium.....	Plasma	Plasma	Plasma	Plasma
Spiral body.....	+	+	+	+

We applied the isolation method by means of plain agar medium for 5 generations, when it was reinoculated into the plasma-ascites medium. This time again growth of the spiral bodies took place with the growth of the *subtilis*. From this it will be seen that not only was it impossible to isolate the spiral bodies from *B. subtilis*, but also that we cannot help thinking that there must be a special relation between the spiral bodies and *B. subtilis*.

In order to clear up the special relation, several strains of *B. subtilis* were inoculated into the plasma-ascites medium in which they

were seen to be mixed with the spiral bodies. We tried to grow various other species of bacteria in the plasma-ascites medium, and have found that with many species of bacteria the spiral bodies were also produced. From the results of these experiments we arrived at the conclusion that all the bacteria that are provided with flagella produce the spiral bodies in their culture. Especially numerous spiral bodies were seen to develop in the culture of *B. subtilis*, *B. tetani*, and *B. anthracis*. Other bacteria produced them in a very small number. Among the bacteria that are lacking flagella, *B. mallei* alone produced the spiral bodies, other bacteria having no flagella were found not to produce spiral bodies. From these experiments, it would seem that the spiral bodies are produced by certain species of bacteria under the certain conditions. We do not hesitate to state that the spiral bodies under consideration are not spirochetes at all.

TABLE 5
BACTERIA PROVIDED WITH FLAGELLA PRODUCE SPIRAL BODIES IN THEIR CULTURE

Species of Bacteria	Appearance of Spiral Bodies in Cultivation	Flagella
1. <i>Staphylococcus</i> I.	—	—
2. <i>Staphylococcus</i> II.	—	—
3. <i>Pneumococcus</i> I.	—	—
4. <i>Pneumococcus</i> II.	—	—
5. <i>Streptococcus</i>	—	—
6. <i>Streptococcus mucosus</i>	—	—
7. <i>Streptococcus mucosus acidilacti</i>	—	—
8. <i>Gonococcus</i>	—	—
9. <i>Diphtheria bacillus</i>	—	—
10. <i>Pseudo-diphtheria bacillus</i> I.	—	—
11. <i>Pseudo-diphtheria bacillus</i> II.	—	—
12. <i>Bacillus bulgaricus</i>	—	—
13. <i>Bacillus anthracis</i>	—	—
14. <i>V. cholerae-gallinarum</i>	—	—
15. <i>B. dysenteriae</i>	—	—
16. <i>B. dysenteriae</i>	—	—
17. <i>B. dysenteriae</i>	—	—
18. <i>B. mallei</i>	+	—
19. <i>B. typhosus</i>	+	+
20. <i>Paratyphoid bacillus</i> A.	+	+
21. <i>Paratyphoid bacillus</i> B.	+	+
22. <i>B. coli</i>	+	+
23. <i>B. proteus</i>	+	+
24. <i>B. typhi-murium</i>	+	+
25. <i>B. tetani</i>	+	+
26. <i>B. pyocyaneus</i>	+	+
27. <i>V. cholerae</i>	+	+
28. <i>B. subtilis</i> (sinegma)	+	+
29. <i>B. subtilis</i> (water)	+	+

As to the transformation of various bacteria into the spiral bodies, we may say that the flagella or a portion of the bacterial bodies can undergo an unusual development under a special circumstance from the following data: (a) the spiral bodies are formed from flagellated

species alone; (b) they show the same reaction toward staining fluids as the flagella; (c) there exist transitional forms from the flagella to the spiral bodies as shown by Zettnow's silver impregnation method. Moreover, by applying our method to the cultivation of bacteria, the flagella may be easily detected, for *B. mallei*, which had been considered to have no flagella, was found to have them.

Here it may be stated that we examined *Spirocheta obermeieri*, and found that it has no flagella, which is contrary to the statement of Zettnow, who affirmed the presence of flagella in that spirochete. We do not, however, insist on our view absolutely because we are not provided with the microphotographic apparatus having 6,000 magnification, with which Zettnow affirmed his view.

SUMMARY

We found spiral bodies resembling spirochetes in anaerobic cultures of various bacteria cultivated in the plasma-ascites medium, Noguchi's ascites-agar and Shimamine's horse serum medium.

The spiral bodies are nothing more than an unusual development of the flagella or parts of the bacterial bodies.

The spiral bodies seem to be identical with Noguchi's involuted forms of *Treponema macrodentium*.

It is suggested from our study that it is necessary to pay special attention to motility, stainability and the pure cultivation in any study of spirochetes, when associated with other bacteria. Morphology alone is not in these cases reliable.

It is further suggested that our method may be applied in the search for flagella. We have discovered the presence of flagella in *B. mallei*, which had been considered to have no flagellum.

EXPLANATION OF PLATE 1

Fig. 1.—Large spiral body.

Fig. 2.—Large spiral body.

Fig. 3.—Large spiral body.

Fig. 4.—Small spiral body.

Fig. 5.—Spiral bodies and the transition forms between the spiral bodies and the flagella as seen in preparations by means of Zettnow's silver impregnation method.



1



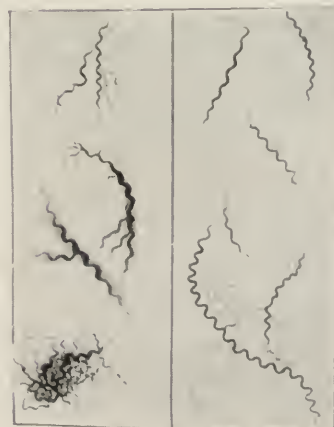
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5

THE BACTERIAL CONTENT OF THE PROSTATE AND ITS RELATION TO PROSTATIC ADENOMA

ROBERT ROSEN

From the James Buchanan Brady Urological Institute, Johns Hopkins Hospital, Baltimore

We have at the present time no adequate explanation of the etiology of tumors, benign or malignant. Irritation, both bacterial and traumatic, is considered by many as an exciting factor, and it was thought interesting to carry out an investigation of the relation of infection to prostatic adenoma.

In this series all the cases selected were those showing prostatic adenoma with the exception of Case 9, which proved to be a prostatic abscess and was included because of its bacteriologic interest.

The only reference found in the literature regarding the rôle bacteria play in prostatic adenoma as obtained from the gland at the operating table is the experiments of Dudgeon and Wallace.¹ They examined the urine, and the prostatic secretion from the gland, and the gland itself, and subjected these to a bacteriologic examination. Their method consisted in obtaining the tissue as soon as the gland was removed, the surface of each specimen was seared with a flat knife, and cultures were made from the interior on blood agar. The prostatic fluid was treated in like manner, and in some cases anaerobic cultures were made. They found the following organisms in their first 14 cases:

Prostate Gland	Cases	Urine	Cases
Sterile	3	Sterile	2
B. coli.....	2	B. coli.....	6
B. coli and diplococcus.....	1	Staphylococcus albus.	5
B. coli and streptococcus.....	1	A diphtheroid bacillus.	1
Staphylococcus albus.....	5		
Staphylococcus albus and streptococcus....	1		
Staphylococcus albus and citreus.....	1		

In another series of 14 cases² they made bacteriologic examination of the prostate gland only and found the following:

	Cases
Sterile	4
Staphylococcus albus.....	3
Staphylococcus albus and other cocci.....	4
B. coli and a diplococcus.....	1
A new pathogenic bacillus.....	1
Streptococcus and B. proteus vulgaris.....	1

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¹ Brit. Med. Jour., 1904, 2, p. 1744.

² Wallace: Prostatic Enlargement, p. 83.

They recovered 28 organisms from 21 of the 28 cases (75%). *Staphylococcus albus* was the commonest organism isolated, and the next most frequent was *B. coli*, while the gonococcus was not once isolated. This is not at all surprising in view of the difficulty of obtaining a suitable medium for the growth of the gonococcus. A point of extreme interest is that in their first series of the 14 cases in which they recovered organisms from both the prostate and the urine, they were always the same, but in some cases an organism was recovered from the gland and none from the urine and vice versa. They conclude that:

Micro-organisms cause a certain amount of inflammation which produces enlargement of the gland, but it is a secondary event, and similar to that which occurs in tumors elsewhere in the body.

That a bacteriologic examination of the urine may throw little or no light in a similar examination of the prostate.

There is no evidence to support the view that enlargement of the gland is of a gonorrheal origin.

Though we attacked the problem with the newer bacteriologic methods, the results were essentially the same.

As this study was undertaken primarily to investigate the etiology of prostatic hypertrophy it is interesting to follow the cycle of the various theories concerning this subject.

DeSault³ was the first to suggest the possibility of prostatic hypertrophy being due to an inflammatory process, basing this view on the observation that it was common to those who had had numerous attacks of gonorrhea. Home,⁴ quoted by Lydston,⁵ suggested the mode of living ("high liver") as one of the predisposing causes. Mercier⁶ believes a sluggish circulation as the factor, while Astley Cooper⁷ says it is a result of old age and is a physiologic condition, this view being also shared by Squier.⁸ Guyon,⁹ Launios,¹⁰ and Regnaud¹¹ believe that it is a senile change, that is, a local manifestation of a general arterio-sclerosis, a fibrosis incident to advancing years, while Casper¹² and Motz¹³ have shown that prostatic hypertrophy can and does exist without sclerosis. Wilson, mentioned by Churchman,¹⁴ cites celibacy on the one hand

³ *Oeuvres Clin.*, 1813, 3, p. 238.

⁴ *Treatment of the Diseases of the Prostate*, 1818, quoted by Lydston.²⁴

⁵ *Etiology of Prostatic Hypertrophy*, 1893.

⁶ *Rech. Anat. Surg. les Mal. d. Org.-Urin. et Gen.*, 1841, p. 218.

⁷ *Lectures on the Genitourinary Organs*, 1824.

⁸ *Med. News*, 1901, 78.

⁹ *Ann. d. mal. d. org. genito-urin.*, 1893, p. 101.

¹⁰ *De l'appareil urinaire de vieillards*, Thèse, 1885, p. 51.

¹¹ *Jour. de l'anat., Par.*, 1892, 28, p. 109.

¹² *Genito-urinary Diseases*.

¹³ *XIII Congr. internat. de med.*, 1900.

¹⁴ *Maryland Med. Jour.*, 1904, 47, p. 400.

and venereal excesses on the other as the probable causes. Amoussat, also quoted by Churchman, considers syphilis as a predisposing factor, and a unique belief is that of Lydston¹⁵ and Civial quoted by Churchman¹⁴ that calculus and stricture may be true causes. A puzzling statement is that of Cabot and Young, Jr.,¹⁶ quoting Cabot and Smith, saying, "that the presence of a previous inflammatory prostatitis prevents later hypertrophy; a stricture and hypertrophy are never present together." Case 31 is in direct opposition to this statement.

Velpeau¹⁷ and the French school consider the enlargement as a neoplastic one; the cause is as unexplained as that of tumors elsewhere in the body, and may be due to an irritation which may itself be due to micro-organisms. This view is the one most adhered to at the present time. Harrison¹⁸ and Daniel¹⁹ conclude that it is a compensatory change in which the bladder is first involved. An extremely interesting belief is that of White,²⁰ shared by Moullin²¹ and Harrison²² who believes there is a relation between the testes and the prostate, based on first, sexual congress, and secondly on the atrophy of the normal prostate after castration in animals (except dogs) and youths. They advocated the therapeutic application of this theory, that is, to castrate to prevent hypertrophy. To offset this theory Moses²³ cites a case of prostatic hypertrophy in a man 68 years old several years after a complete castration, showing that prostatic hypertrophy is independent of the presence or the absence of the testes.

In his admirable work, Ciechanowski,²⁴ showed that changes occurred simultaneously in glandular and stromal portions of the prostate gland and that inflammation is the direct cause (and that this was gonorrhea), as was brought out by his pathologic studies, demonstrating the presence of small round cell infiltration. The glandular change was due to a dilatation of the glandular tubules and not to the formation of new ones. In support of this view are Herring,²⁵ Gouley,²⁶ Dainiel, Barnette,²⁷ Finger,²⁸ and others. Green and Brooks²⁹ in 1902 publish their results. They selected 58 cases for histologic studies, 6 of them were obtained from operations and the rest from necropsies, and they conclude that the inflammatory theory is the true one, and that true neoplasms of the prostates are rare. Two years later Rothschild³⁰ had examined 30 prostates removed from the cadavers of patients who had died between the ages of 34 and 52 without evidence of history of disease of the genito-urinary tract. In 27 he found changes similar to those

¹⁵ Am. Jour. Urol., 1908, 4, p. 453.

¹⁶ Ref. Handbook Med. Sc., 1907-8, 7, p. 323.

¹⁷ Leçons orales de clinique de chir., 1841, 3, p. 478.

¹⁸ Brit. Med. Jour., 1895, 2, p. 1605; *ibid.*, 1886, 2, p. 438; *Lancet*, 1899, 2, p. 126.

¹⁹ *Ibid.*, 1904, 2, p. 1140.

²⁰ *Ibid.*, 1894, 1, p. 1353.

²¹ *Lancet*, 1896, 1, p. 288.

²² *Ibid.*, 1900, 2, p. 96.

²³ *Therap. Monatsh.*, December, 1895.

²⁴ *Anat. Untersuch. u. die 590*, "Prostate Hypertrophie" u. versus proz. M. III. aus der med. u. chir., 1900, 5, p. 183; *Ann. d. mal. d. org. gen.-urin.*, 1901, 19, p. 523.

²⁵ *Brit. Med. Jour.*, 1904, 2, p. 1136.

²⁶ *Jour. Cutan. and Gen.-Urin. Dis.*, 1898.

²⁷ *Med. News*, 1904, 85, p. 863.

²⁸ *Wien. med. Wchnschr.*, 1890, 182, 200, 224, 263.

²⁹ *Jour. Am. Med. Assn.*, 1902, 38, p. 1051.

³⁰ *Centralbl. f. d. Krankh. d. Harn u. Sex. Org.*, 1904, 15, p. 177.

demonstrated by Ciechanowski and Finger in gonorrheal prostatitis, and he infers that the anlage of prostatic hypertrophy is laid down years before the gland actually begins to enlarge and that gonorrhea is at least the usual cause. Grandon,³¹ in his splendid review of the literature of prostatic hypertrophy concludes that the process is a slow formation of connective tissue due to infection, chiefly gonorrheal, as it is the most common, and excludes all other inflammatory causes because of their rarity.

Lydston³⁵ gives as the cause of prostatic hypertrophy:

1. Inflammation—infectious or traumatic.
2. Chronic prostatic hyperemia and sexual irregularities and excesses.
3. Transpelvic infection by the colon bacillus.
4. Calculi.
5. Senile prostatitis.

Dudgeon and Wallace¹ inclined toward the inflammatory theory, but later Wallace² accepted the neoplastic theory as the true one. Rovsing³² believes that there is first a hypertrophy resulting from hyperemia and that in this chronic process there is destruction of the epithelium and the formation of connective tissue resulting in a shrinkage of the gland, and that the hypertrophy is a compensation, while the qualitative deterioration of the secretion is compensated by the quantitative increase. He showed that of 140 patients with the symptoms of prostatic hypertrophy 40 gave a history of gonorrhea.

From a clinical standpoint Keys,³³ Cunningham and Watson,³⁴ Churchman, Casper and Legneu³⁵ are opposed to the inflammatory theory. Posner³⁶ concludes from his studies that the fat, that is, the lecithin, exerts a chemotactic irritation on the white blood corpuscles, and he claims a relationship between chronic prostatitis and hypertrophy, but believes that hypertrophy is primary and that the inflammatory change is secondary to it, even without infection. Goldberg³⁷ expresses a similar view. He found the secretion of a patient with prostatic hypertrophy who never had gonorrhea and had never been catheterized, to contain pus cells in the secretion which was scanty and difficult to obtain.

These views, so conflicting in character, apparently are difficult to conciliate; and yet it is considered possible that not any of these theories is the correct one, but that a combination of several may be the exciting causes.

In the early part of my experiments for the purpose of studying the bacterial content of the prostate I devised a method for surface sterilization and concluded that 10 seconds in paraffin oil at 180 C. would be sufficient to destroy surface organisms and permit one to draw definite conclusions from glandular bacterial studies. In those

³¹ Ann. Surg., 1902, 36, p. 813.

³² Archiv. f. klin. Chir., 1904, 74, Part 4.

³³ Jour. Am. Med. Assn., 1904, 43, p. 187.

³⁴ Watson and Cunningham.

³⁵ Traite Surg. d'urol.

³⁶ Am. Jour. Urol., 1904, I, p. 215.

³⁷ Folio Urologica, 1907.

experiments, the staphylococcus was used as a control on the method employed, but later, using *B. coli* as a control, growth was obtained; that is, 10 seconds at 180 C. in paraffin oil was not sufficient to kill *B. coli*. The thermal deathpoint of the colon bacillus was later determined and found to be 20 seconds. Knowing that *B. coli* is thermostabile, the 20 seconds was accepted as the length of time required to sterilize the surface of glandular tissue, as is seen in Table 1.

As the field of operation is in close proximity to the rectum, and to secure pieces of tissue from the living body (especially this region) without contamination of the surface of these pieces is beyond doubt extraordinarily difficult, we feel that 20 seconds should be used, for though one undoubtedly runs a risk of destroying thermolabile organisms within the tissue, yet if a less effective method is used it is impossible to be certain that surface contamination has not influenced the results, as can easily be seen from the cases in which a control was made to determine that the time of surface sterilization was sufficient to destroy all the surface organisms. I did not begin this until the 31st case, at which time I had proven the technic to be effective. The results up to that time can only be of small interest and any conclusion drawn must be accepted with much reserve.

TECHNIC

A sterile glass jar containing an evaporating dish, scissors and forceps are kept ready, and as soon as the operator enucleates a prostatic lobe, the lid is removed from the jar and the tissue is dropped by the operator or the assistant into the jar. This is carried to the laboratory and emulsified in the sterile air chamber as follows:

Hands are scrubbed, washed and soaked in bichlorid solution, introduced into the gloves of the sterile air chamber, the mortar is put underneath the opening, into which 25 cc of dextrose ascitic broth is poured. The flange is flamed and closed; the oil, which is in a tall narrow pyrex beaker to prevent ebullition, is heated up to 185 C. and allowed to come down to 180 C.; the tissue is immersed in the oil for 20 seconds after which it (tissue and mouse teeth clamp) is dropped into the mortar after flaming the flange, there to be emulsified and poured into a large test tube. To check this method, a piece of autoclaved kidney was dipped in a 24-hour broth culture of staphylococci (later *B. coli* was used), heated, as the prostatic tissue was, and run through a similar process. The emulsions were then removed from the chamber and anaerobic and aerobic cultures were made, using hydrocele or ascitic fluid, and the emulsions were then put in the refrigerator for future use. Pieces of prostatic tissue were transferred from the emulsion to the culture tubes, and sealed with paraffin to facilitate anaerobic conditions, and kept in the incubator for at least 3 weeks before discarding. One cc of the emulsion was plated at once. The time of appearance and general character of the colonies, their distribution and subcultures made on various

media, both aerobically and anaerobically, were noted. In most cases the pathologic report as made by the resident pathologist and their interpretation are given.

Beginning with the twelfth case it was considered advisable to run a control to note the organisms contaminating the surface as received directly from the operator. As soon as the tissue was brought to the laboratory 10 c c of broth was poured over the gland and allowed to remain 5-10 minutes, 1 c c of this being then plated. From here on the technic was the same as before. In Table 2 a complete analysis of the cases is tabulated.

TABLE 1
PRELIMINARY EXPERIMENTS ON STERILIZATION OF SURFACE OF TISSUE AFTER INOCULATION

Length of Time Tissue Was Heated in Oil at 180 C.							
10 Sec.	12 Sec.	15 Sec.	18 Sec.	20 Sec.	22 Sec.	25 Sec.	30 Sec.
+	+	+	+	+	0	0	0
+	+	+	+	+	0	0	0
+	+	+	+	0	0	0	0
+		0	0	+	0	0	0
+		0	0	0	0	0	0
+		0	0	0	0	0	0
+		0	0	0	0	0	0
+		0	0	0	0	0	0
0		0		0	0	0	
0		0		0			
0		0		0			
0		+		0			
+		0		0			
+		0		0			
+		0		0			
+		+		0			
+		+					
+		+					
+		+					
+		0					
		+					
		+					
		+					
		0					

Explanation: + = growth; 0 = sterile plate.

DISCUSSION

From Table 1 it is seen that the thermal deathpoint of *B. coli* lies between 15 and 20 seconds in paraffin oil at 180 C. We also see why in several of the control experiments, 15 seconds was sufficient to kill *B. coli* in Cases 27 and 28, for we find a similar condition in these experiments. Again we see that 10 seconds seemed to be sufficient to kill *B. coli* in 6 series. For these divergent results no definite explanation can be offered other than that the organism may have been of a different strain; of lower resistance; fewer in number or that the size of the tissue used may have varied; or a combination of

these factors may account for the results. It is certain that 25 seconds will not kill some organisms that may be on the interior of the gland, as can be seen in Cases 31, 32 and 33 from which staphylococci were isolated. I selected 20 seconds as the time to destroy *B. coli* on the exterior. When this time was employed 3 showed positive growth out of 18 cases.

From this series of 39 cases it is seen in Tables 2 and 3 that 14 organisms have been isolated (36%). More organisms were recovered but they cannot be included in the group, that is, as coming from the prostatic tissue proper, because the technic was not sufficiently developed until the 31st case. I did include those cases in which the plate poured immediately after the emulsion was made showed innumerable colonies; also where there was a co-existing abscess, and in which the surface organism was of one type while that isolated from the emulsions was of another kind.

The fact that more bacteria were not isolated may be explained by the fact that the organism either had died out in the tissue or had become attenuated and thermolabile and the only signs of inflammation were the small round cell infiltration.

As regards the relationship between prostatic hypertrophy and gonococcus infection, Table 2 shows that of the 39 cases in this series 29 patients (74.7%) denied infection, while only 10 admitted (20.3%), so that the anamnesis shows that individuals with a negative history are as susceptible, if not more so, to prostatic hypertrophy, as those who have had a gonococcus infection. In the 4 patients from whom an organism was isolated not one admitted having gonorrhea.

The micro-organisms found on the surface of the gland, in 29 of the cases, were for the most part similar to those isolated from the prostatic emulsion. In several cases, however, Cases 18, 31, 32, 39 and 40, the organisms were different. In Cases 13, 21 and 24 no surface organisms were found. In the last case it is peculiarly interesting to note that while the surface culture did not yield an organism, the prostatic emulsion yielded a facultative anaerobic gram-positive staphylococcus, so that the surface organism need not in all cases be considered as contaminators; for, in some cases, the bacteria may be in the prostate gland and may be exposed by the cutting of the tissue. In urine no attempt was made to determine the type of organism present as done by Wallace and Dudgeon, other than whether it was a coccus or a bacillus.

TABLE 2
RESULTS OF CULTURES

Case	Gonorrhea	Urinalysis	Method of Surface Sterilization		Organisms Isolated	Surface Organisms		Control on Method		Pathologic Diagnosis	Remarks
			Medium	Time		No.	Type	Time	Org.		
1	Denied	Coccus and bacillus	Salt solution	15 sec.	G.* Streptococcus? Streptobacillus? 0	1. Round cell infiltration. 2. Benign hypertrophy, fibroglandular. 3. Chronic prostatitis.	Method of sterilization is questionable here.
2	Denied	Coccus and bacillus	Flame. Water not sterilized	15 sec.	B. coli? B. coli	1. Round cell infiltration. 2. Benign hypertrophy, fibroglandular.	Contamination?
3	Denied	Bacillus	Flame	Passed through flame several times	B. coli	Marked peri-acinus small round cell infiltration. P. H., F. O.; chr. prostat.	This organism we believe to have been on the interior of the gland.
4	Denied	Coccus and bacillus 0	Flame, water	8 times, 20 sec.	0	No round cell infiltration. P. H., F. G.	No organisms and no round cell infiltration.
5	Denied	0	Oil	5 sec.	0	5 sec.	Staphylococcus	Organism from swab of seminal vesicles lost in transferring.
6	30 years ago	Coccus and bacillus	Water	7 sec.	0	7 sec.	Staphylococcus	Round cell infiltration. P. H., F. G.; chr. prostatitis.	No organism isolated. Control contaminated, so time is lengthened in next case.
7	Denied	Bacillus	Water	10 sec.	0	10 sec.	Staphylococcus	Marked round cell infiltration. P. H., F. G.; chr. and subacute prostatitis.	No organism isolated. Control positive growth.
8	46 years ago	Coccus	Water	10 sec.	0	10 sec.	Staphylococcus	Marked round cell infiltration. P. H., F. G.; chr. prostatitis.	No organisms isolated.
9	10 weeks ago	0	B. coli communis	This case was one of prostatic abscess and B. coli communis was isolated.
10	25 years ago	Bacillus	Paraffin oil	10 sec.	0	10 sec.	Staphylococcus	Moderate grade of round cell infiltration. P. H.; chronic prostatitis.	Here an organism should have been isolated, if any relation is to be drawn from the pathological sections unless it was very thermolabile.
11	Denied	Bacillus	P. O. 180 C.	10 sec.	0	10 sec.	Staphylococcus	Moderate grade of small round cell infiltration. P. H., F. G.; chr. prostatitis.	Here an organism should have been isolated, if any relation is to be drawn from the pathological sections unless it was very thermolabile.

12	Denied	Coccus and bacillus	P. O. 180 C. Saline solution	6 sec. 20 sec.	B. coli?	2,530	1. B. coli 2. Staphylococcus albus 3. Streptococcus	6 sec.	Staphylococcus	0	Slight grade of round cell infiltration. P. H., F. G.; chr. prostat.	We cannot accept the B. coli without question in this case, for the surface was well covered with organisms. We only obtained it from the oil method, while flame and 20 sec. in saline was negative. On account of B. coli in previous case it was used as a check to control method in this case.
13	Several times	0	P. O. 180 C.	10 sec.	00	0	0	10 sec.	B. coli	+	Slight grade of small round cell infiltration. P. H., F. G. type.	Again we see that 10 sec. was not sufficient to completely sterilize the surface of gland.
14	Denied	Bacillus	P. O. 180 C.	6 sec. 10 sec. 15 sec.	B. coli communior ?	1,500	B. coli communior; Staphylococcus albus; Streptococcus	10 sec.	B. coli	+	Marked round cell infiltration. P. H.; chr. prostatitis	
15	Denied	0	P. O. 180 C.	8 sec.	B. coli communis	3,000	B. coli communis	8 sec.	Staphylococcus	0	Round cell infiltration. P. H., glandular abscess.	B. coli communis was isolated from interior as well as surface.
16	Denied	Coccus and bacillus	P. O. 180 C.	10 sec.	B. coli communis	B. coli	10 sec.	Staphylococcus	0	Moderate grade of small round cell infiltration. P. H., fibro-cystic type; chronic prostatitis.	B. coli was isolated but cannot be accepted as in gland proper; method was inefficient.
17	Denied	Bacillus	P. O. 180 C.	10 sec.	0	B. coli	10 sec.	Staphylococcus	0	Moderate grade of small round cell infiltration. P. H., glandular; chr. prostatitis.	This case is interesting for 10 sec. seemed to destroy the B. coli and can only explain it on the fact that there were only a few organisms.
18	Denied	Coccus	P. O. 180 C.	10 sec. 15 sec.	B. welchii 0	200	Staphylococcus albus	10 sec.	Staphylococcus	0	Slight grade of small round cell infiltration. P. H., F. G. type.	Isolated B. welchii, but believe it to be a contaminator.
19	30 years ago Denied	Coccus bacilli Bacilli	P. O. 180 C.	10 sec.	0	100	Staphylococcus albus	10 sec.	Staphylococcus	0	Marked small round cell infiltration.	No organisms isolated.
20	Denied		P. O. 180 C.	10 sec.	Gas producing organism. Anaerobic 0	5,000	?	10 sec.	Staphylococcus	0	Moderate amount of small round cell infiltration. P. H., F. G.; chr. prostatitis.	Failed to isolate organisms.
21	Denied	Bacillus and cocci	P. O. 180 C.	10 sec.	0	0	10 sec.	Staphylococcus	0	Marked grade of small round cell infiltration. P. H., F. G.; chr. prostatitis.	This is the case in which the surface culture was negative, and the section showed a marked round cell infiltration. Control again was ineffective.
22	Denied	Coccus	P. O. 180 C.	10 sec.	0	550	Staphylococcus aureus and albus	10 sec.	B. coli	+	Moderate grade of small round cell infiltration. P. H., F. G. type.	
23	20 years ago	Bacillus	P. O. 180 C.	10 sec.	Staphylococcus	4,000	Staphylococcus albus	10 sec.	B. coli	+	Slight grade of small round cell infiltration. P. H., F. G. type.	There is no question about the organism being from the gland but the control is again found at odds.

TABLE 2—Continued
RESULTS OF CULTURES

Case	Gonorrhea	Urinalysis	Method of Surface Sterilization		Organisms Isolated	Surface Organisms		Control on Method		Pathologic Diagnosis	Remarks
			Medium	Time		No.	Type	Time	Org.	Result	
24	Denied	No record	P. O. 180 C.	10 sec.	Facultative anaerobic staphylococcus; gram-positive	0	0	10 sec.	Staphylococcus	0	This is the 3rd case in which the surface culture was negative, isolated gram-positive staphylococcus, facultative anaerobe.
25	Denied	Bacillus	P. O. 180 C.	10 sec.	0	700	B. coli	10 sec.	B. coli	0	This case complicated our method by 10-sec. in P. O. at 180 C. which was sufficient to destroy B. coli.
26	Denied	Coccus and bacillus	P. O. 180 C.	10 sec.	B. coli	0	B. coli	10 sec.	B. coli	+	B. coli was isolated from gland in spite of the positive control.
27	Several	No record	P. O. 180 C.	15 sec.	B. coli?	3,750	B. coli	15 sec.	B. coli	0	Though we believe the organism isolated is from the gland proper, we cannot include it as such owing to the surface culture and later work on the T. D. P. B. coli.
28	Denied	Coccus and bacillus	P. O. 180 C.	15 sec.	Bacilli, belonging to the colon group	10,000	?	15 sec.	B. coli	0	The organism isolated belonged to the colon group.
29	Denied	Streptococcus; staphylococcus; bacillus	P. O. 180 C.	15 sec.	0	60	Streptothrix	15 sec.	B. coli	+	The control showed growth up-setting previous results.
30	At 50	Bacillus	P. O. 180 C.	15 sec.	B. coli communis?	360	B. coli	15 sec.	B. coli	+	The control was again positive.
31	No history of N	Coccus and bacillus	P. O. 180 C.	25 sec.	B. fecalis alkaligenes	20	B. coli; staphylococcus albus	25 sec.	B. coli	0	There is no question about this organism being indigenous to the gland. In this case we find stricture and hypertrophy together.
32	Denied	Coccus	P. O.	25 sec.	Anaerobic bacilli failed to isolate	1,000	1. B. coli. 2. Staphylococcus. 3. Unknown bacilli.	25 sec.	B. coli	0	Unable to isolate anaerobic bacillus.

33	Denied	Bacillus	P. O.	25 sec.	Staphylococcus; B. pyocyaneus	10,000	1. B. coli. 2. Staphylococcus. 3. B. pyocyaneus.	25 sec.	B. coli	0	Moderate grade of small round cell infiltration. P. H., G.; prostatic abscess.	There was a prostatic abscess associated with the hypertrophy. A staphylococcus and pyocaneus was isolated showing that 25 sec. will not destroy some organisms on the interior of the gland. Isolated 2 organisms out of 3, which appeared in surface culture. We are certain the coc- cus was isolated from the gland, but do not feel the same about the bacillus. Organism isolated was evidently from the interior of the gland as the control remained sterile. The organism B. coli was on surface of gland.
34	Denied	Coccus and bacillus	P. O.	20 sec.	1. Staphylococcus 2. B. Proteus vulgaris?	50	1. B. coli. 2. Staphylococcus	20 sec.	B. coli	0	Marked grade of small round cell infiltration. P. H., F. G. type; chronic prostatitis.	
35	Denied	Coccus and bacillus	P. O.	20 sec.	B. coli	5,000	B. coli	20 sec.	B. coli	0	Marked grade of small round cell infiltration. P. H., G. type; chronic prostatitis.	
36	Denied	Bacillus	P. O.	20 sec.	0	200	B. coli	20 sec.	B. coli	0	Moderate grade of small round cell infiltration. P. H., F. G.; chronic prostatitis.	
37	Denied	0	P. O.	20 sec.	0	1,500	Staphylococcus aureus	20 sec.	B. coli	0	Marked grade of small round cell infiltration. P. H., G.; chronic prostatitis.	The reason no organism was isolated may be because it was thermolabile.
38	Denied	Bacillus	P. O.	20 sec.	0	2,000	Staphylococcus aureus	20 sec.	B. coli	0	Marked grade of small round cell infiltration. P. H., F. G.; chr. prostatitis.	As in previous case organisms may have been a thermolabile one.
39	Denied	Bacillus	P. O.	20 sec.	B. coli communis, 0 (spore bearer G.)	10,000	Staphylococcus albus	20 sec.	B. coli	0	Slight grade of small round cell infiltration. P. H., G. cystic type.	The surface culture showed staphylococcus and was not obtained from the gland emulsion from which B. coli communis was recovered with a G. + spore-bearing bacillus (contamination).
40	Admitted	Mixed coccus	P. O.	20 sec.	Facultative anaerobe bacillus, G., non-motile	5,000	B. coli. Staphylococcus albus	20 sec.	B. coli	0	Marked grade of small round cell infiltration. P. H., G.; chronic prostatitis.	The organism was indigenous to the gland and shows 20 sec. will kill B. coli infection.

* P. H.—Prostatic hypertrophy. F.—Fibroid type. G.—Glandular type. F. G.—Fibro-glandular type. P. O.—Paraffin oil.

TABLE 3
ORGANISMS ISOLATED FROM 39 CASES OF THE PROSTATE GLAND

Type of Organism	Found Alone	Associated with Other Organisms	Gonococcus Infection Denied	Gonococcus Infection
<i>B. coli</i>	5		5	
<i>Staphylococcus</i>	1	2	2	
Colon-like bacillus	1		1	
Anaerobic gas forming organism.....	1		1	
Facultative anaerobe, gram-positive staphylococcus	1		1	
<i>B. fecalis alkaligenes</i>	1		1	
Anaerobic bacilli failed to isolate.....	1		1	
<i>B. pyocyaneus</i>	0	1?		
<i>B. proteus vulgaris</i>	0	1?		
Unknown bacillus. Facultative anaerobe, gram-negative, G., nonmotile	1		1	

Case 9 was a prostatic abscess and is not included in above table.

TABLE 4
SURFACE ORGANISMS ISOLATED FROM 29 CASES OF THE PROSTATE GLAND

Type of Organism	Found Alone	Associated with Other Organisms
Sterile	3 times	
<i>B. coli communis</i>	9 times	6 times
<i>B. coli communior</i>		1 time
<i>Staphylococcus albus</i>	4 times	8 times
<i>Staphylococcus aureus</i>	2 times	1 time
<i>Streptococcus</i>		2 times
<i>Streptothrix</i>	1 time	
<i>Streptobacillus</i>		1 time
Unknown bacillus		1 time
<i>B. pyocyaneus</i>		1 time
<i>B. proteus vulgaris</i>		1 time
Unknown	2 times	

SUMMARY

Ten seconds is not sufficient to destroy all surface organisms as stated in a previous article.³⁸

Twenty seconds in paraffin oil at 180 C. is sufficient to kill *B. coli* and permit the staphylococcus to be isolated from the interior of the gland, if present, but may destroy more thermolabile organisms.

Organisms were isolated from 14 cases (36%).

The surface and interior organisms were the same in some cases; the former need not necessarily be a contaminator.

There does not seem to be any relationship between the small round cell infiltration, as is seen in the sections of each gland, and the organism isolated. The cellular reaction was present in all but one case in this series.

³⁸ Davis and Rosen: Jour. Infect. Dis., 1917, 21, p. 323.

The colon group of organisms was the commonest found; no one organism was found specific to the gland; not once was the gonococcus isolated.

The history of the cases in this series shows that persons with a negative record as to gonococcus infection to be as susceptible to prostatic hypertrophy as those with infection.

No comparison can be drawn between the urinary organisms and those isolated from the gland.

The significance of the bacteria isolated from the prostate gland and the rôle that they may play in prostatic hypertrophy, if any (other than that they may be secondary invaders), cannot be determined from this investigation.

AGGLUTINATION IN MEASLES

RUTH TUNNICLIFF (Chicago)

Contract Surgeon, U. S. Army

In a previous article¹ I have described a small, gram-positive diplococcus, which was isolated in anaerobic cultures from the blood of measles patients, during the pre-eruptive and early eruptive stages of the disease. Similar diplococci were also isolated from the throat, nose, eye, and in one instance from the ear.

During the acute stage of measles, I found that the serum showed a diminished opsonic power for this organism,² followed by a marked increase as the symptoms subsided and the rash began to fade. This increase in opsonins occurred generally on the third or fourth day after the appearance of the eruption and was specific for the measles diplococcus. The serum of measles patients gave slight complement fixation for the diplococcus,³ at the time corresponding to the height of the opsonic reaction.

Measles serum was found also to agglutinate this diplococcus, but when first isolated the organism often agglutinated so much spontaneously that no satisfactory experiments could be made. Later, when studying the serum of rabbits immunized with diplococci isolated from measles, it was found³ that strains about one year old grew diffusely in dextrose broth and did not agglutinate spontaneously, but were agglutinated distinctly by immune rabbit serum. Hence it seemed worth while to study again agglutination reactions in measles. The new experiments were made at Camp Meade, Md. The serum of 10 measles patients was examined, generally daily, for agglutinins by the macroscopic method. A strain, isolated from the blood of a measles patient a few hours before the appearance of the eruption, was used in the experiments. Another blood strain isolated at the same time of the disease, was also tested from time to time and found to correspond closely in its agglutinative reaction. The diplococci were transplanted from human blood agar to dextrose (2%) broth and incubated 24 hours. The mixtures of diluted serum and broth culture

¹ Jour. Am. Med. Assn., 1917, 68, p. 1028.

² Jour. Infect. Dis., 1918, 22, p. 462.

³ Tunnicliff and Brown, Jour. Infec. Dis., 1918, p. 572.

were incubated at 37 C. for 2 hours and kept in a refrigerator 18 hours, when the results were read. As a rule, no agglutinins are present in the serum on the second day after the appearance of the eruption. With the disappearance of the symptoms and the rash, a distinct increase of agglutinins occurs on the third to the eighth day, most often on the third or fourth, the agglutinins persisting from 1-7 days. Agglutination occurs only in low dilutions, no serum agglutinating in a dilution above 1:8. A person vaccinated with the diplococci 6 months previously showed agglutinins at 1:2 with two different measles blood strains.

TABLE 1
AGGLUTININ REACTION IN MEASLES

Case	Day of Disease after First Appearance of Rash													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1		1:2	1:8	1:4		1:2		1:2						0
2				1:4	0		0							
3		0	0	0	0	1:8		1:8		0		0		
4					1:4			0						
5			1:4		0									
6			1:4	0	0									
7		0	0	0	1:2	1:2	0	0						
8		0	1:4	1:2	0	0								
9		0	1:8	1:4	0	0								
10		0	0	1:4	1:8	1:2	1:2	0	0					

0 = no agglutination.

Figures = highest dilution of serum causing distinct agglutination.

None of the normal serums used as controls agglutinated the diplococcus. The serum of a patient with bronchopneumonia and the serums of 4 influenza patients were examined, generally daily, with the measles diplococcus but no agglutination was observed. Agglutination tests with active measles serum and pneumococci types I and II and *Streptococcus hemolyticus* were negative.

These experiments show, then, that a distinct increase in agglutinins for the diplococci isolated from measles blood occurs in measles on the third to the eighth day following the appearance of the eruption. The agglutination appears to be specific for the measles diplococcus, no agglutinins being observed for pneumococci types I and II, or *Streptococcus hemolyticus*. The agglutinins are present in the blood when the opsonic power is at its height and when complement fixing bodies are demonstrable.

A SIMPLE METHOD FOR ISOLATION OF INFLUENZA BACILLUS

E. P. BERNSTEIN AND L. LOEWE

From the Pathological Laboratory of the Mount Sinai Hospital, New York

The great difficulty in the isolation of the influenza bacillus from the respiratory tract is due to the fact that the accompanying bacteria usually overgrow and obscure the organism. This statement is borne out by the first studies that we made of cultures taken from the tonsils and the nasopharynx of 12 patients suffering from influenza or influenza accompanied with pneumonia. The material obtained was streaked on human blood-agar plates, and though colonies of the influenza bacillus developed in all but one, the subsequent isolation in pure culture was extremely difficult, none being pure on the first subculture.

We also examined the sputums from 59 cases of pneumonia following influenza, and though we were able to demonstrate the presence of the influenza bacillus in mixture with other organisms by direct smears in 15 instances, but 10 showed these organisms in the primary culture and only one was obtained pure in the first subculture.

These discouraging results led us to seek for a method which would favor the growth of the influenza bacillus in the presence of accompanying bacteria.

In 1912 Churchman demonstrated the selective action of gentian-violet on bacteria. He showed that agars containing this dye in strengths of 1:100,000 to 1:1,000 were either inhibitive or destructive to gram-positive organisms, while gram-negative bacteria were not affected. With this in mind we developed a method for obtaining the influenza bacillus in pure culture from mixtures containing it.

The usual accompanying organisms in the naso-pharynx, trachea or lungs are the pneumococcus, streptococcus and staphylococcus, all of which are gram-positive and therefore inhibited by gentian-violet.

After many experiments with blood-agar containing varying amounts of the dye, we found that a concentration of 1:5,000 gave the best results. Agar was prepared with a H ion of 7.1 and a gentian-violet content of 1:5,000—taking a concentrated alcoholic solution of the dye as unity. At first we prepared our medium by

adding to the melted and cooled gentian-violet-agar 25% of sterile ascitic fluid and 5% of sterile defibrinated human blood. We soon found that the serum interfered with the inhibiting action of the gentian-violet for the gram-positive organisms. It was therefore eliminated.

TABLE 1
SHOWING THE ADVANTAGE OF USING GENTIAN-VIOLET-BLOOD-AGAR IN
ISOLATING THE INFLUENZA BACILLUS

No.	Gram Stain	Blood-Agar		Gentian-Violet Blood-Agar	
		Primary Culture	Subculture	Primary Culture	Subculture
1	Influenza bacillus; G.+ chains	Hemolytic streptococcus; influenza bacillus	Influenza bacillus, pure	Influenza bacillus; streptococcus	Influenza bacillus, pure
2	Influenza bacillus; G.+ diplococci and chains	Pneumococcus; streptococcus	Influenza bacilli not present	Influenza bacillus; streptococcus; pneumococcus	Influenza bacillus, pure
3	G.+ diplococci and chains; influenza bacillus	Hemolytic streptococcus; pneumococcus; influenza bacillus (few)	Influenza bacillus, not pure	Influenza bacillus; streptococcus; pneumococcus	Influenza bacillus, pure
4	Influenza bacillus; G.—large bacillus; G.+ diplococcus	Pneumococcus Type IV; bacillus lactis aerogenes; influenza bacillus	Influenza bacillus, not pure	Bacillus lactis aerogenes; influenza bacillus; pneumococcus	Influenza bacillus, pure
5	G.+ diplococci and clusters influenza bacillus	Staphylococcus albus; pneumococcus mucosus Type III; influenza bacillus	Influenza bacillus, not pure	Influenza bacillus; pneumococcus; staphylococcus	Influenza bacillus, pure
6	G.+ diplococcus; influenza bacillus	Pneumococcus; influenza bacillus	Influenza bacillus, not pure	Influenza bacillus; pneumococcus	Influenza bacillus, pure
7	G.+ diplococci; chains and clusters; influenza bacillus	Pneumococcus Type IV; staphylococcus albus; influenza bacillus	Influenza bacillus, not pure	Influenza bacillus; pneumococcus; staphylococcus	Influenza bacillus, pure
8	G.+ diplococci and chains; influenza bacillus	Pneumococcus; hemolytic streptococcus; staphylococcus albus	Influenza bacilli not present	Pneumococcus; staphylococcus; influenza bacillus; streptococcus	Influenza bacillus, pure
9	G.+ diplococci and chains; influenza bacillus	Hemolytic streptococcus; influenza bacillus; pneumococcus	Influenza bacillus, not pure	Influenza bacillus; streptococcus; pneumococcus	Influenza bacillus, pure

The gentian-violet blood-agar was poured into sterile plates and stored for use in the refrigerator. Surface streaks were made from washed sputum or from the naso-pharynx by means of tubes.

While the pneumococcus, streptococcus and staphylococcus were not entirely eliminated from the primary plate, they were as a rule so much inhibited in their growth that influenza bacillus colonies were easily seen and recognized. Control plates on plain blood agar were

often so overgrown by the accompanying bacteria as to make the isolation of the influenza bacillus impossible.

Subcultures on gentian-violet blood-agar usually completely inhibited associated gram-positive organisms. If still present, a second subculture on the same medium produced a pure culture of influenza bacillus. As shown by Table 1, we cultured 9 sputums in duplicate — on plain blood-agar and on gentian-violet blood-agar. In one case the influenza bacillus was isolated by both methods in the same generation and in the other 8 cases it was obtained pure in the second or third generation only on gentian violet plates, and in only a few instances in later subcultures on plain blood plates.

SUMMARY

By the employment of gentian violet blood agar the isolation of the influenza bacillus is comparatively easy. The use of this medium gives a much higher percentage of positive results than plain blood agar.

CHEMICAL CHANGES IN TUBERCULOUS TISSUES

GEORGE T. CALDWELL

From the Department of Pathology of the University of Chicago and the Otho S. A. Sprague Memorial Institute, Chicago.

In this study of the chemical composition of tuberculous tissues, the lack of uniformity in the methods used by different investigators made it imperative, for the sake of comparative results, to make analyses of normal as well as tuberculous tissues. Since large specimens were required, bovine tissues were used chiefly as large amounts of tuberculous material were available in an extensive packing center. Lymph gland and liver tubercles were used exclusively for the tuberculous specimens and normal lymph glands and livers furnished the materials for comparison. Such a comparative study reveals some of the more marked changes in the tissues, resulting from the reaction to the tubercle bacillus.

Numerous more or less complete analyses of the livers of men and of animals have been reported. The water and the fats contained in the liver received the greater share of attention from many of the earlier workers. v. Bibra¹ as early as 1849 gave 76.19% as the average water content of 6 approximately normal human livers. The amount of fat in these livers is given as 2.86% of the moist weight, or about 12% of the dry weight. Similar determinations on bovine livers gave the water content of 2 specimens as 70.86 and 71.92%, with fat percentages of 2.64 and 3.28, or calculated on the dry weight, the average percentage of fat is 10.35. In a more recent study of normal bovine liver, Profitlich² reported the average percentage of water in 7 different livers as 71.66. The fat content of these livers seems to have varied widely, ranging from 10.87-21.78% of the dry weight, with an average of 16.75%. The ash determined for one of these livers constituted 3.83% of the dry weight.

In connection with his study of the human liver in acute yellow atrophy and in chloroform poisoning, Wells³ made careful analyses also of the normal human liver. He gives 77.6% as the amount of water in the normal human liver and 5% of fat. Lecithin and cholesterol determinations were made on the lipin fractions obtained from the livers of 2 persons who died suddenly. The lecithin averaged 31.7% of the ether-soluble substance, or 6.3% of the dry weight; cholesterol formed 6.7% of the ether-soluble material, or about 1.3% of the dry weight. The total amount of lecithin in the liver in acute yellow atrophy was very greatly reduced, not only as to the actual amount present, but also in relation to the other constituents of the liver. It formed

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¹ Quoted by Von Gorup-Besanez, *Lehrbuch d. physiol. Chem.*, 1878, p. 711.

² *Arch. f. d. ges. Physiol.*, 1907, 119, p. 465.

³ *Jour. Exper. Med.*, 1907, 9, p. 627; *Jour. Biol. Chem.*, 1908-9, 5, p. 129.

17.6% of the ether-soluble substances, or 2.9% of the dry weight. A decrease in lecithin was also noted in delayed chloroform poisoning, although fatty changes were moderate in degree, but the decrease was by no means so marked as in acute yellow atrophy. The amount of cholesterol in the liver in acute yellow atrophy was not so greatly reduced, in fact, the total amount for the entire liver was about the same as that found in the controls, although it constituted a larger percentage of the ether-soluble substances. This is accounted for by the reduction in the amounts of neutral fats and lecithin.

In chloroform necrosis, the increase in the fat content of the liver was found to be due entirely to simple fats. As a continuation of the analyses of human liver, Wells studied also the alcohol-ether-insoluble fraction. The residue left after extraction with alcohol and ether was extracted in a shaking machine repeatedly with fresh quantities of water, and then with water at 50-60 C. The residue of liver tissue was then extracted with boiling water. The amount of gelatin in the hot water extract was determined and the remaining solids, obtained by evaporating the filtrate, were added to the united cold and warm water extracts. After the concentration of these extracts, the proteose-peptone mixture was precipitated by means of alcohol. Amino-acids and purins were determined in the nonprotein portions of the watery extracts. The residue of insoluble liver substance left after all the extractions had been completed, was dried to constant weight and analyzed. The composition of the coagulated and insoluble proteins of the liver after thorough extraction with alcohol, ether, cold and hot water, was found to be quite the same in chloroform necrosis and in acute yellow atrophy as in normal livers.

Determinations of insoluble sulphur, phosphorus and iron were made on the extracted residues. The sulphur was practically constant in all 4 specimens, in spite of the great structural changes in the 2 diseased livers. The insoluble phosphorus, however, in the acute atrophy and chloroform necrosis livers was, in each instance, increased to about 4 times the amount present in the normal livers.

The increase in the phosphorus in acute yellow atrophy is explained as the result of the great proliferative activity exhibited by the cells of the stroma and bile ducts in areas where regeneration is taking place, giving rise to large numbers of new cells rich in nucleic acid. The phosphorus found in the normal livers constituted 0.24% of the extracted residue, in acute yellow atrophy and in chloroform necrosis the phosphorus was 0.90% of this residue. The amount of sulphur ranged from 0.75-0.82% of the extracted material in the normal and pathologic livers. Heffter⁴ studying the liver in relation to phosphorus poisoning, made lecithin determinations on the livers of normal rabbits and found that it constituted about 2.2% of the dry weight. In another series of rabbits which had been poisoned with phosphorus, the average lecithin content dropped to 1.12%, or about $\frac{1}{2}$ that of normal livers. Baskoff⁵ made lecithin and jecorin determinations on normal dog livers and on the livers of dogs poisoned with alcohol. In the normal animals, the phosphatids constituted 8.4% of the dry weight, while in animals which had been poisoned with alcohol for 9 months, the phosphatids had decreased to 3.9%. The jecorin in the normal livers was 14.4% of the total phosphatids, while in the animals poisoned with alcohol it had increased to 39%.

⁴ Arch. f. exp. Path. u. Pharm., 1891, 38, p. 97.

⁵ Ztschr. f. physiol. Chem., 1909, 62, p. 162.

⁶ Quoted by von Gorup-Besanez, Lehrbuch d. physiol. Chem., 1878, p. 732.

Normal lymph glands, either of human beings or of the lower animals, have evidently never been made the subject of as careful studies as those recounted for the liver. In the inguinal glands of an old woman, Oidtmann⁶ reported the finding of 71.43% of water, 28.45% of organic material and 0.12% of inorganic substances. In the mesenteric lymph glands of oxen, Bang⁷ reported 80.41% of water, 19.59% of solids, 13.79% of total proteins, 0.69% of histone nucleinate, 1.06% of nucleoprotein, 4.76% of substances soluble in alcohol and 1.05% of mineral constituents. The structurally related thymus gland has been subjected to accurate chemical studies but here the chief interest has usually been centered in the nuclein substances. Lilienfeld⁸ analyzed the cells of the calf thymus and reported a total phosphorus content of 3.01% of the dry weight; the total nitrogen was 15.03%, lecithin 7.51%, cholesterol 4.40%, fat 4.02%, and the silver salts of the nuclein bases 15.17% of the dry weight. The dry weight of these cells amounted to 11.49% of the fresh weight. An analysis of the human spleen by Burow⁹ and Magnus-Levy¹⁰ showed the water content of that organ to be 78.4%, the solids 21.53%, the fat 2.77% and nitrogen 2.79%, all calculations being made on the fresh weight of the organ. A complete analysis of the dog spleen was made by Corper.¹¹ The averages of the analyses of 3 normal spleens gave a moisture content of about 75-77% and a content of ether-soluble materials between 11.6 and 15.5% of the dry weight. The ether-soluble fraction was made up of about 1.5% of cholesterol, and between 6 and 7% of lecithin, leaving 2-6.5% for neutral fats. The total soluble nitrogen ranged between 0.45 and 0.97% of the dry weight and was about equally divided between that precipitable with tannic acid and that which could not be so precipitated. The water-soluble phosphorus content was about 0.27-0.52%. With the amounts of tissue used, no purins could be identified in the water-soluble fraction. The insoluble part of the tissue contained about 0.26-0.98% of dry weight as iron, 0.53-0.60% as sulphur and about 0.39% as phosphorus, with a purin nitrogen content of 0.24-0.35% of the dry weight. The total nitrogen content of the insoluble part was about 11-13% of the dry weight. Of the purin enzymes, evidence was obtained of the presence of xanthin oxidase, adenase and guanase, while uricase was lacking.

The nature of the changes occurring in tissues during the caseation produced by infections with the tubercle bacillus has been the subject of much speculation and has led to numerous investigations limited chiefly to the fatty constituents. On the basis of the gross appearance and, likewise, from the ordinary microscopic preparations, it seemed evident that caseous material was rich in fats; that it consisted chiefly of globules of fat and granules of coagulated protein. It was thought, perhaps, that the tubercle bacillus which seems to be able to synthesize fat when grown on glycerin agar, might produce somewhat similar changes in the necrotic area and its vicinity, giving rise in this way to the fatty changes of caseation.

Schmaus and Albrecht,¹² having studied caseation necrosis microscopically, state that the process consists of the death of the cellular elements and the origin of a firm intercellular substance arising as a transudate, together with the precipitation of a fibrinoid material, probably not identical with fibrin but giving the typical reaction of fibrin. The formation of the caseous detritus

⁷ Hofmeister's Beiträge, 1903, 4, p. 115.

⁸ Ztschr. f. physiol. Chem., 1894, 18, p. 473.

⁹ Biochem. Ztschr., 1910, 25, p. 165.

¹⁰ Biochem. Ztschr., 1910, 24, p. 363.

¹¹ Jour. Biol. Chem., 1912, 11, p. 27.

¹² Virchows Arch., 1896, 144, p. 72, Suppl.

follows by a progressive breaking up of the fibrinoid material. To these substances are attributed the chief importance in the formation of the firm dry condition of the caseous mass. Along with the production of the intercellular substance goes the disappearance of the chromatin of the cell nuclei. Using fat stains, Rosenthal¹³ found no evidence of fat in miliary tubercles unless there was some caseation present. The fat seems to make its appearance with the occurrence of the caseation. Within an extensive caseous area no fat was found, or at most only traces, while just at the boundary of the caseous area, fat-bearing cells were seen. Some of the giant cells in this region are said to resemble fat cells, while other giant cells were apparently fat-free. Streaks containing small fat droplets were sometimes seen in sections cut through fresh caseous areas. These are explained as representing the boundaries of small caseous areas which have fused to form the larger area, in which case the fat had not disappeared as it seems to do with the gradual extension of the caseation in the tuberculous tissue. Likewise, Vallillo,¹⁴ in studying avian tuberculosis, observed that non-necrotic tubercles composed of epithelioid and giant cells contain either no stainable fat or only sparsely grouped fat droplets in the center. In the tubercles which had necrotic centers, the fat droplets were abundant and were located chiefly in the cytoplasm of the giant cells accumulated there. The fat droplets, however, were not numerous in the caseous part of the tubercles.

Similar observations were made by Hagemeister¹⁵. He noted that in large conglomerate tubercles, fat droplets were not infrequently observed within the tuberculous area close to the margin of the older individual tubercles. In other cases, the boundary zone apparently contained a large amount of fat. Sometimes, in the caseous tubercles, two or even three of these fat-containing marginal zones were noted at regular intervals, an occurrence explained as being due to the advance of the necrosis in successive stages. Even in the necrotic areas the outlines of the giant cells could sometimes be made out by the arrangement of the fat droplets. Herxheimer,¹⁶ studying lung and lymph gland tubercles for the occurrence of fat, confirmed the findings of Rosenthal and Hagemeister. Chaussé,¹⁷ using sudan III and osmic acid, demonstrated fat droplets in the giant cells and in practically all other cells in the tuberculous area and also in the substance derived from the destruction of all these cells. In a study of necrobiotic fatty changes, Joest¹⁸ used fat stains on tuberculous tissues with results quite similar to those previously reported by Rosenthal. He was never able to demonstrate fat in the intercellular substances. Emphasis is placed on the observation that although fat is demonstrable in the caseous part of the tubercle, it is less prominent there than in the boundary zone of the living tissue, and the living tubercle tissue apart from the boundary zone is free from stainable fat. It is explained, however, that this impression of a lesser fat content in the caseous portion is, doubtlessly, due in part to the fact that the fat in the living cells of the boundary zone occurs in sharply circumscribed globules, while, in the caseous area, the globules are broken up and the fat more diffusely scattered.

¹³ Verhandl. d. deutsch. path. Gesellsch., 1899, 2, p. 440.

¹⁴ La Tuberculosi, IV, 257, 1911 2.

¹⁵ Virchows Arch., 1903, 172, p. 72.

¹⁶ Ergeb. d. allg. Path. u. path. Anat., 1902, 8, p. 669.

¹⁷ Compt. rend. Soc. de biol., 1909, 64, p. 377.

¹⁸ Virchows Arch., 1911, 203, p. 451.

¹⁹ Jour. Med. Research, 1905 6, 14, p. 491.

In the study of pathologic calcification, Wells¹⁹ used both staining and chemical methods for the recognition of fats in tuberculous tissues. Specimens of human and bovine tuberculous lymph glands, stained with sudan III, revealed marked infiltration with fine and coarse fat granules all through the areas that are acellular, the largest and most abundant granules being usually at the periphery. When counterstained with hematoxylin, the calcium deposits were found to lie in such tissues as were stained for fat, but there was no particular difference to be noted in the amount or character of the fat in the vicinity of the calcium deposits and elsewhere. Not infrequently a calcium deposit was noted at the periphery of the gland or tubercle, while, in the center, there was no calcium but many fat granules, although not more than in the tissues surrounding the calcium deposits. For the chemical studies, Wells used tuberculous mediastinal lymph glands of cattle. He calls attention to the fact that bovine tuberculous lesions differ from human lesions in that calcification occurs during the progress of the disease and is extensive in the form of innumerable sandlike granules, scattered all through the tuberculous tissue even while the disease is in the most active stages. Calcification is usually an evidence of latency or healing in human tuberculous areas, and the deposits are found in much larger masses, each of which usually corresponds to an entire tubercle. Two sorts of bovine material were collected and examined separately in the course of his investigations. One consisted of the fluid puslike content of the large softened glands. This material escapes when the glands are opened and contains but few granules of calcium large enough to be felt by the finger. The other specimen was obtained by scraping the surface of unsoftened tubercles and the walls of the tubercle cavities. It consisted largely of the calcified material and the adherent tissue, mixed with more or less of the tissue elements but giving a fair conception of the substances immediately about the calcified masses. After drying this tuberculous material, it was thoroughly extracted with ether, alcohol and amyl alcohol. The inorganic salts of calcium were quite insoluble in these solvents. The residues left from these extractions were then extracted with large volumes of water and calcium, magnesium, phosphorus and carbon dioxid determinations made on the water-insoluble residues. The total lipin content of the scrapings from the walls of the calcified bovine lymph glands was found to be very appreciably higher than that of the caseous liquid content of these lymph gland tubercles. Since these values, in either case, are based on the dry weight, this difference is dependent in no direct way on the water content. Likewise, the low lipin content of this dried caseous material cannot be attributed to the presence of the heavy calcium salts, for both the calcium and the phosphorus are present in only about $\frac{1}{3}$ of the amount found in the scrapings from the tubercle walls. The lipin content of calcified human tuberculous lymph glands is low, but in this case the calcium and phosphorus values are extremely high, showing that the dry weight here is made up in large part of calcium salts. The MgO was found to hold a constant relation to the CaO and the amount present was always small; likewise, a rather definite ratio existed between the amounts of carbon dioxid and of CaO. The water-soluble fraction of the caseous liquid content of the tubercles constituted a smaller percentage of the dry weight than it did in the scrapings from the walls of these tubercles. The water-soluble materials obtained from the calcified human tuberculous glands was still much smaller in amount. In the latter case this might be due to the presence of great amounts of relatively insoluble inorganic salts.

²⁰ Zur Chemie der Verfettung, Dissertation, Basel, 1902. Quoted by E. Schmoll, Deutsch. Arch. f. klin. Med., 1904, 81, p. 163.

Perhaps the most complete of the analyses which have been made of the lipin fraction in tuberculous caseous material are those which were made by Bossart.²⁰ The materials used were of human origin and only 1 consisted of completely caseous material, the remaining 4 specimens ranged from $\frac{1}{6}$ - $\frac{1}{3}$ caseous substances obtained from lymph glands. The reported fat content in percentage of dry weight varied from 13.77 in a specimen which was estimated as $\frac{1}{6}$ caseous to 23.79 in a specimen $\frac{1}{4}$ caseous. The total fat content of the pure caseous material is given as 20.75% of the dry weight. The figures reported for the lecithin content are apparently of no great value since no lecithin at all was obtained in 3 out of the 5 specimens. In 3 specimens varying from $\frac{1}{6}$ - $\frac{1}{3}$ caseous, cholesterol ranged from 25.8-33.5% of the total fats, while the latter are reported as varying from 13.77-15.73% of the dry weight. In the completely caseous specimen, the cholesterol value is given as 2.77% of the alcohol extract, apparently a much smaller amount of cholesterol than that found in the partially caseous specimens. The negative findings for lecithin reported by Bossart have not been substantiated by other workers with caseous material. Schmoll²¹ was able to demonstrate considerable amounts of glycerol-phosphoric acid in the alcoholic extracts of all specimens of pure caseous material which he examined. He used 3 specimens of completely caseous material from bovine lymph glands, and 1 specimen of human material which was about $\frac{1}{4}$ caseous. The caseous residues left after alcohol-ether extraction were extracted with cold water with the addition of toluene. This was found to be a very troublesome procedure as it was necessary to change the water twice daily for 5 or 6 weeks in order to make the extractions complete. No protein materials seemed to go into solution, as all the protein reactions were either completely negative or scarcely evident. Elementary analyses were made on the residue insoluble in alcohol, ether and water. The calculations made on the basis of the ash-free material gave the following averages for the 3 specimens of completely caseous material: carbon, 53.92%; hydrogen, 7.38%; nitrogen, 16.44%, and sulphur, 0.65%. The ash content varied from 9.2-23.3%. Phosphorus was determined only on the specimen of caseous material containing 23.3% ash and in this case it constituted 1.04% of the ash-free substance. The ash value for the human tuberculous specimen, $\frac{1}{4}$ caseous, was 4.63%, with a phosphorus content of only 0.25%. This exceptionally low percentage of phosphorus is stated as being surprising, since it was obtained from tuberculous but not completely caseated lymph glands. As this tissue is normally rich in phosphorus, this finding seemed to indicate that the process of coagulation necrosis, as appears evident also microscopically, is accompanied by a destruction of the cell nuclei and a washing away of the products formed. Whatever the sulphur content means, it was thought to be distinctly lower here than in most proteins. However, the value given is slightly higher than that given by Corper¹¹ for the sulphur content of the dog spleen. In order to find out more concerning the character of the protein in caseous material, Schmoll studied its conduct toward pepsin-HCl digestion and hydrolytic cleavage with HCl. The fluid obtained by about 2 months' digestion with the pepsin-HCl mixture showed that this protein substance differs in no essential way from other proteins so far as its conduct toward digestion is concerned. From the results obtained, Schmoll thought that he could exclude with certainty the existence of any nuclein material whatever, since

²¹ Deutsch. Arch. f. klin. Med., 1904, 81, p. 163.

no precipitate was obtained with ammoniacal silver solution and he remarks that the undissolved portion was certainly not nuclear material. Following hydrolysis with HCl, a partition of the nitrogen gave the following values:

Humin and ammonia nitrogen.....	5.01% of total N
Basic nitrogen.....	43.9 %
Amino-acid nitrogen.....	51.1 %

The low percentage of humin and ammonia nitrogen as well as the richness in basic nitrogen is noted as being remarkable. An attempt to determine guanine and adenine was unsuccessful. Schmoll studied also the autolysis of tuberculous caseous material and found that the autolytic processes go on extremely slowly in such tissues. He remarks that this may explain the fact that caseous material is so rarely absorbed. In connection with F. Müller, Schmoll analyzed the lipin fraction of a specimen of caseous material from human lymph glands. Cholesterol was found present but the amount is not stated. The phosphorus content of the ether-soluble material was 1.57%, or when calculated as lecithin, 38.31%. This corresponds to 3.83% of the dried caseous material.

A study of the lipoids and their content in phosphorus in different organs and tissues of guinea-pigs, during chronic tuberculosis, was made by Griniew.²² He finds that in this disease the composition of the cells of nearly all the organs and tissues changes so far as the lipoid content is concerned. The change is qualitative as well as quantitative and is shown by the diminution in the amount of phosphorus in the lipoids and by the replacement of some lipoids by others. The total quantity of all the lipoids decreases as well. The quantity of cholesterol is increased in the liver, kidney, brain and heart, and decreased in muscles, lung, spleen and bone marrow. There is less lecithin than normal in all the organs, that is, it constitutes a smaller percentage of the total lipins. In nearly all of the organs, the percentage of cephalin is increased. The enzymes of the tuberculous tissues were also studied and the lipolytic power of the tuberculous organs was apparently subnormal. The lungs, liver and kidneys are reported as markedly subnormal in catalase, while the catalase content of the heart is increased.

A comparison of normal human livers with those of 5 persons dying of tuberculosis was made by Robin.²³ The water content was approximately 7.7% higher in the tuberculous livers than in the normal ones and this change seemed to be more marked in the acute than in the chronic forms of tuberculosis. There was only a slight change in the fat content and a minimal lessening of organic sulphur.

Corper²⁴ used intra vitam staining methods in a study of the fat in the tubercles of guinea-pigs. It had been shown by the work of Riddle²⁵ and others that fat dyes such as sudan III and scarlet R, entering the body dissolved in fat, remain either entirely or chiefly with this same food fat, being deposited with it if the food fat was deposited, but not leaving the food fat to enter either stored fat or the intracellular fats or lipoids of active tissues. Corper observed that the fats of tubercles never contained any demonstrable amount of the fat dyes administered, no matter whether the tubercles formed before or after the animal was saturated with the dye. He states, therefore,

²² Arch. des sciences biol., 1912, 17, p. 177 and p. 363.

²³ Ztschr. f. d. ges. Physiol. d. Stoffwechs., 1911, 6, p. 576.

²⁴ Jour. Infect. Dis., 1912, 11, p. 373.

²⁵ J. Exper. Zool., 1910, 8, p. 163.

that it seems probable that the fats microscopically visible or chemically demonstrable in tubercles, are derived chiefly or solely from the existing fats and lipoids of the disintegrated cells and are not deposited from the fats in the blood. This view is entirely in harmony with the histologic evidence.

EXPERIMENTAL

Through the courtesy of the Western Packing Company and the Peerless Packing Company of Chicago, large amounts of bovine lymph gland and liver tubercles were obtained, and also fresh normal lymph gland and liver. Both the normal and the tuberculous lymph glands came from the peribronchial and mesenteric regions. The normal peribronchial lymph glands were of the usual size for cattle, the largest being about 6 cm. in greatest dimension. The mesenteric lymphoid tissue occurred in two forms: (1) lymph glands of the usual rounded or oval form with a definite hilus, and (2) long strips of lymphoid tissue, sometimes 20-30 cm. long, about 1 cm. wide and of a corresponding thickness.

From all of these lymph glands, the surrounding fatty tissues were removed with great care until no definite masses of fat were left anywhere on the surfaces of these glands. The fibrous capsules, however, were not removed and, undoubtedly, an appreciable amount of fat was left unremoved. This is noteworthy here, since the method of preparing the specimens from the tuberculous glands was such that this extraneous fat would not enter to contaminate the tuberculous materials, since, in no case, were the tuberculous glands used entirely, but only the walls of the tubercles and the caseous materials from these tubercles. The tubercles in the lymph glands averaged from 3-5 cm. in diameter and their caseous centers were sometimes 2-3 cm. in diameter. The surrounding normal tissues were removed as completely as possible from the tubercles, after which the tubercles were opened and their caseous contents expressed. The walls of the peribronchial lymph gland tubercles were kept separate from the walls of the mesenteric lymph gland tubercles, while the caseous material from all of these lymph gland tubercles was made into one composite specimen, since the amount was not sufficient to divide.

Likewise, the tubercles occurring in bovine livers were separated carefully from the surrounding liver tissue and the caseous material removed from the surrounding fibrous capsule. The largest of these tubercles varied from 1-5 cm. in diameter, while some of the livers were studded elsewhere with many smaller tubercles.

Only the larger tubercles which could be more readily separated from the normal tissues were used for these analyses. Two specimens of caseous material were obtained from tubercles having a diameter of 1-2 cm., and one specimen came from tubercles 2-5 cm. in diameter. Two specimens consisting of liver tubercle walls were preserved for analysis.

The normal and tuberculous tissues were all obtained fresh from the packing house, and all were treated throughout in as nearly as possible the same manner. The normal tissues and the walls of the tubercles were ground fine in a meat chopper, after which samples were removed for the determination of the water content and the dry weight. The caseous material consisted of a semifluid mass in which there was no macroscopic evidence of calcification other than the occurrence of numerous sandlike particles which tended readily to settle toward the bottom of the container leaving the superficial layers more distinctly fluid. The caseous material from the largest liver tubercles was more watery in its appearance than that from the smaller tubercles. Samples of the caseous material were, likewise, removed for the determination of the dry weight. The specimens of the normal and the tuberculous tissues, selected in such a way as to contain approximately 100 gm., were then weighed and about 5 volumes of 95% alcohol, containing a minimum of nonvolatile substances, were added. The specimens were then placed on a steam bath where they were allowed to remain for an hour at a temperature closely approximating the boiling point of the alcohol. Some of the specimens were used for immediate analysis, while the others preserved as indicated were kept for subsequent use.

General Plan of Analysis.—(a) Water content and dry weight of tissues.

Amounts of the freshly ground tissue, varying from 0.5-1.5 gm., were weighed in appropriate weighing bottles and, after some preliminary drying at a lower temperature, were placed in an electric oven which was maintained at a temperature of 90-100 F. A practically constant weight was obtained by heating 48-72 hours. Some of the tissues in the weighing bottles were first treated with several volumes of 95% alcohol, which partially dissolved out the fats and rendered the tissue more porous so that a constant weight could be obtained with a briefer period of heating.

(b) Extraction of lipins.

When one of the preserved specimens was selected for analysis, it was first heated on the steam bath and the supernatant alcohol containing a part of the lipins was filtered through the extraction cup which was to be subsequently used in the Greene extraction apparatus. The specimen was extracted 3 times in this way with redistilled 95% alcohol. The residue was then placed on a watch glass and allowed to dry, first at room temperature and then in

the electric oven at a temperature of 90-100 F. The dried tissue was then ground in a mortar and transferred to the extraction cup which had been used previously as a filter. It was next extracted for 24 hours with hot absolute alcohol and then for 24 hours with redistilled ether. The residue was then removed from the extraction cup and the ether allowed to evaporate. The residue so obtained was once more ground in a mortar and the powdered tissue again extracted for 24 hours with absolute alcohol. All of these extraction fluids were united in a measuring flask of 500 or 1,000 cc capacity. In the early part of this work all of this lipin solution was evaporated to dryness at a temperature of 50-60 C. under reduced pressure and the total lipin fraction determined by weighing the residue so obtained. When this procedure was followed, after a nearly constant weight had been obtained in a vacuum desiccator, this lipin fraction was emulsified in water and transferred to a measuring flask of 500 or 1,000 cc, depending on the amount of fatty material obtained. Most of the lipin substances were readily transferred to the flask by means of the water emulsion; the remainder was dissolved in chloroform, which was added in small amounts up to a total volume of 25 cc. With the volume of the emulsion so adjusted that it nearly $\frac{3}{4}$ filled the flask, 10 cc of concentrated hydrochloric acid were added from a pipet while the contents of the flask were being rotated and thoroughly mixed. This usually caused a complete breaking up of the emulsion, the lipins settling to the bottom of the flask in the chloroform solution. The flask was then filled to the mark and allowed to stand until the lipins had settled out leaving a clear fluid. The supernatant acid fluid was then filtered through paper, care being taken to prevent the chloroform solution from leaving the flask. The lipin-chloroform mixture was then dissolved in hot 95% alcohol and made up to volume. This alcoholic solution of the lipins is the one which was subsequently used for the determination of cholesterol, lecithins and the iodine number. The acid solution obtained by decantation from the lipin chloroform mixture constitutes what is here called the water-lipin fraction. The volume of this fraction was noted and phosphorus and nitrogen determinations made on aliquot parts of it. The values so obtained were used in making corrections for the water-lipin solution retained by the lipin-chloroform mixture. All the phosphorus present in this water-lipin solution was considered to be inorganic phosphorus. With some of the specimens of normal tissue, the entire lipin fraction was not dried to constant weight, but instead an aliquot part, usually $\frac{1}{10}$, was used for this purpose and a similar fraction was removed for determination of the iodine number. The remainder was evaporated to a syrup on the water bath and then emulsified with water as above indicated.

(c) The alcohol-ether-insoluble fraction consists of the dried residue left after extraction with alcohol and ether.

Small amounts of this residue were used for ash, calcium, total nitrogen and total phosphorus determinations. The remainder which constituted 0.7 or 0.8 of the entire amount was preserved for subsequent extraction with water.

(d) Water-soluble fraction.

The water-lipin solution was neutralized with sodium hydroxide and a definite portion of this used to extract a corresponding portion of the alcohol-ether residue. Either 0.7 or 0.8 of the total amount was used in each case. The neutralized water-lipin solution was divided into 2 equal parts and each in turn was used to extract the alcohol-ether residue. This extraction was accomplished in a shaking machine, each period of shaking lasting for 2 hours. Two subsequent extractions were, likewise, made using each time approxi-

mately 300 cc of distilled water. The suspended particles were removed from these extraction fluids by centrifugalizing. All four of these fluids were united, made slightly acid with acetic acid and evaporated to 1,000 cc. This constitutes the so-called water-soluble fraction, aliquot parts of which were used for the determination of the different forms of nitrogen and of phosphorus.

(c) Water-insoluble fraction.

This fraction is made up of the residue left after the completion of the alcohol, ether, and water extractions. It was used for the determination of total nitrogen, total phosphorus, phosphoprotein phosphorus, ash, calcium, and finally for the determination of the purin nitrogen.

ANALYTICAL METHODS

The determinations of cholesterol were made by Corper's method²⁶ while lecithin was estimated by the method of Koch and Woods,²⁷ the phosphorus being determined finally as $\text{Mg}_2\text{P}_2\text{O}_7$. The lecithin value is obtained by multiplying that of the phosphorus by the factor 25.75 on the assumption that the molecular weight of the lecithin is approximately 800. The iodine numbers of the lipin fractions were obtained by the use of von Hübl's iodine solution, the excess of iodine being titrated with 0.1 N $\text{Na}_2\text{S}_2\text{O}_3$. All total nitrogen determinations were made by the Kjeldahl method. For the estimation of the proteoses in the water soluble fraction, the proteoses were precipitated by saturation with ZnSO_4 in a solution made acid with H_2SO_4 . The amount of nitrogen in this precipitate was then determined by the Kjeldahl method. The free amino-acids in the water-soluble fraction were estimated by the Van Slyke method.²⁸ For the peptones and peptids, a part of the solution was completely hydrolyzed and the total amino-acid content determined; from this total amino-acid nitrogen, the nitrogen of the free amino-acids and of the proteoses was subtracted. The negative value which was often obtained when these deductions were made serves to illustrate the unsatisfactory nature of this method of nitrogen partition. For all total phosphorus determinations, a Neumann combustion²⁹ was performed and the phosphorus ultimately determined gravimetrically as $\text{Mg}_2\text{P}_2\text{O}_7$.

The inorganic phosphorus was precipitated from the water soluble fraction by means of magnesia mixture, and the phosphorus determined gravimetrically as in other cases. An attempt was made to split off the phosphoprotein phosphorus in the water insoluble frac-

²⁶ Jour. Biol. Chem., 1912, 12, p. 197.

²⁷ Jour. Biol. Chem., 1905-6, 1, p. 203.

²⁸ Jour. Biol. Chem., 1913, 16, p. 121; and 1915, 23, p. 408.

²⁹ Ztschr. f. physiol. Chem., 1904, 43, p. 32.

tion by means of the action of 1% NaOH. From the solution so obtained, the phosphorus was determined in the same way as the inorganic phosphorus. The ash obtained from the alcohol-ether residues and from the water-insoluble fractions was analyzed for calcium by McCrudden's method.³⁰ The greater part of the water-insoluble fraction was completely hydrolyzed with 5% H₂SO₄, and the purin nitrogen estimated by the method of Krüger and Salomen.³¹

RESULTS OF ANALYSES

A comparison was made of the water content and dry weight of the normal tissues with the water content and dry weight of the caseous material and the walls of the tubercles arising from these tissues.

TABLE 1
BOVINE LYMPH GLANDS AND LYMPH GLAND TUBERCLES

Source of Tissue	Nature of Specimen	Water Content % of Moist Weight	Dry Weight % of Moist Weight
Peribronchial lymph glands.....	Normal	82.37	17.63
Mesenteric lymph glands.....	Normal	81.44	18.56
Mesenteric lymph glands.....	Normal	81.74	18.26
Peribronchial.....	Walls of tubercles	79.95	20.05
Peribronchial.....	Walls of tubercles	78.56	21.44
Peribronchial.....	Walls of tubercles	79.41	20.59
Mesenteric lymph glands.....	Walls of tubercles	79.51	20.49
Mesenteric lymph glands.....	Walls of tubercles	79.28	20.72
Peribronchial and mesenteric.....	Caseous material	75.16	24.84

From Table 1, it is seen that, in every case, the water content of the normal lymph glands is distinctly higher than that of the tuberculous tissues. The lower water content of the walls of these lymph gland tubercles is, doubtlessly, due in part to the replacement of the succulent normal tissue by a dense fibrous connective tissue, and, in part also, to the early deposition of calcium salts in the caseous material included within these tissues. Even the semifluid caseous material from the lymph gland tubercles has a distinctly lower water content than either the normal lymph glands or the walls of the tubercles. This is readily understood, however, when it is found that this caseous material has an ash content of about 25% of the dry weight. The presence of the heavy inorganic salts accounts for the high value for the dry weight and the corresponding low water content.

³⁰ Jour. Biol. Chem., 1911, 10, p. 187.

³¹ Hoppe-Seyler-Thierfelder, Handbuch d. physiol. u. pathol. chem. Analyse, 1909, p. 188.

As compared with normal bovine lymph gland tissue, the water content of normal bovine liver is distinctly lower, the average in the lymph glands analyzed being 81.85% as contrasted with an average of 70.68% for liver tissue. This value for the liver is distinctly lower than that obtained from the dense fibrous walls of lymph gland tubercles, so it might be expected that when liver tissue is destroyed in tubercle formation and new connective tissue takes its place, that the water content of the tubercle walls would be higher than that of the normal tissue. This is found to be the case. The average water content of the tubercle walls was 76.93% as compared with 70.68% for the normal liver tissue. In the walls of the lymph gland tubercles, the percentage of water present was 79.35, a value approximating that obtained for the walls of liver tubercles much more closely than do the percentages obtained for the two normal tissues.

TABLE 2

WATER CONTENT AND DRY WEIGHT OF NORMAL BOVINE LIVER COMPARED WITH THE WATER CONTENT AND DRY WEIGHT OF THE CASEOUS MATERIAL AND WALLS OF TUBERCLES FROM BOVINE LIVERS

Source of Tissue	Nature of Specimen	Water Content % of Moist Weight	Dry Weight % of Moist Weight
Liver.....	Normal.....	70.60	29.40
Liver.....	Normal.....	70.67	29.33
Liver tubercles.....	Walls of tubercles.....	77.07	22.93
Liver tubercles.....	Walls of tubercles.....	76.80	23.20
Liver tubercles.....	Caseous material.....	73.00	27.00
Liver tubercles.....	Caseous material.....	74.73	25.27
Liver tubercles.....	Caseous material from large tubercles.....	78.45	21.55

The amount of water in the caseous material from the liver tubercles varies considerably in the different specimens, the extreme variation being from 73.00-78.45%. This latter value was obtained with the caseous material from only the largest of the liver tubercles, all of which were over 2 cm. in diameter. The caseous centers of these large tubercles were observed to be much more fluid than the smaller ones. The average water content for the 3 specimens of caseous material from the liver tubercles is 75.39% which agrees very closely with the percentage of water present in the single specimen of caseous material from lymph gland tubercles, namely, 75.16.

The average total lipin content obtained for the 3 separate determinations on normal bovine lymph gland tissue is 24.39% of the dry weight, or 4.4% of the moist weight. The peribronchial lymph glands

gave a somewhat lower value for the total lipins than that obtained from the mesenteric glands. This difference may be explained, at least in part, by the fact that the surrounding fat could be more completely removed from the peribronchial glands. The surfaces of the mesenteric glands were always distinctly oily even after a careful removal of the closely clinging fat tissue.

TABLE 3

TOTAL LIPIN FRACTION FROM NORMAL BOVINE LYMPH GLANDS AND FROM LYMPH GLAND TUBERCLES

Source of Tissue	Nature of Specimen	Moist Weight of Specimen	Dry Weight of Specimen	Dry Weight in % of Moist Weight	Total Lipins		
					Weight in Grams	% of Dry Weight	% of Ash-free Residue
Peribronchial lymph glands.....	Normal	74.4	13.117	17.63	2,980	22.72	23.81
Mesenteric lymph glands.....	Normal	100.7	18.730	18.60	4.588	24.50	25.80
Mesenteric lymph glands..... (Long form)	Normal	100.7	18.317	18.19	4.756	25.96	27.29
Peribronchial lymph glands.....	Walls of tubercles	102.8	20.61	20.05	6.140	29.79	32.61
Peribronchial lymph glands.....	Walls of tubercles	102.4	21.08	20.59	6.268	29.73	32.92
Peribronchial lymph glands.....	Walls of tubercles	106.9	22.92	21.44	6.607	28.80	31.26
Mesenteric lymph glands.....	Walls of tubercles	101.2	20.74	20.49	6.180	29.80	32.21
Mesenteric lymph glands.....	Walls of tubercles	63.3	13.12	20.72	3.830	29.19	31.44
Peribronchial and mesenteric lymph glands	Caseous material	87.8	21.81	24.84	4.250	19.49	27.05

The fat content of the tubercle walls is strikingly constant in the 5 specimens here studied, the maximum variation being 1% of the dry weight. The average fat content of the walls of these lymph gland tubercles is 29.46% of the dry weight, as compared with 24.39% for normal lymph glands. This is an increase of more than 20%. This finding, if considered alone, would seem to substantiate the claim made on the basis of fat staining that it is the cells at the boundary of the necrotic portion of the tubercle which are especially rich in fat. This relationship is not at all definite in the liver tubercles. The caseous material from the lymph gland tubercles, on the other hand, is found to contain a much lower percentage of lipins than occurs in the walls of these tubercles. The total lipins in this specimen constituted 19.49% of the dry weight, while the average for the tubercle walls was 29.46%, or approximately $\frac{1}{2}$ more. The lipin content of the caseous material was even distinctly less than was that of the normal lymph gland tissue, the average for the normal tissue being 24.39% as compared with 19.49% in the caseous material.

This is a decrease of 20% below the normal value when the calculations are made on the dry weight. However, the dry weight of the caseous material is made up in large part of calcium salts. The ash content of this specimen is about 28% as compared with 5% in the normal tissue, while the nitrogen content of the alcohol-ether insoluble fraction of the caseous material is only 67% of the nitrogen content of the normal tissue. If the calculations were made on the basis of the organic constituents of the caseous substance, there would be an actual increase in lipins in the caseous material when compared with normal lymph glands.

TABLE 4
TOTAL LIPIN CONTENT OF NORMAL BOVINE LIVER AND LIVER TUBERCLES

Source of Tissue	Nature of Specimen	Moist Weight of Specimen	Dry Weight of Specimen	Dry Weight in % of Moist Weight	Total Lipins		
					Weight in Grams	% of Dry Weight	% of Ash-free Residue
Liver.....	Normal	101.0	29.694	29.40	10.204	34.36	35.07
Liver.....	Normal	100.1	29.249	29.22	9.762	33.38	34.16
Liver.....	Normal	101.2	29.682	29.33	10.329	34.77	35.55
Liver tubercles.....	Walls of tubercles	167.6	38.421	22.93	5.848	15.23	16.68
Liver tubercles.....	Walls of tubercles	105.4	24.453	23.20	3.939	16.11	17.68
Liver tubercles.....	Caseous material	109.1	29.457	27.00	5.147	17.47	24.70
Liver tubercles.....	Caseous material	109.3	27.620	25.27	4.875	17.65	23.70
Liver tubercles.....	Caseous material large tubercles	101.7	21.916	21.55	3.971	18.12	22.74

The total lipin content of normal bovine liver forms a higher percentage of the dry weight than in normal lymph glands. The average of 3 determinations on specimens all of which came from the same liver was 34.17% of the dry weight. Three parallel determinations made on specimens from another bovine liver gave a total lipin content of 31.28%. For the 2 livers the mean value is 32.72% of the dry weight, or 9.6% of the moist weight. When compared with the normal tissue, the walls of the liver tubercles have a remarkably low content of fatty material. The average for the 2 determinations made is 15.67% of the dry weight, or less than half the amount of fat obtained from the normal tissue. This result forms a striking contrast with that obtained by extraction of the walls of lymph gland tubercles, in which the fat content was definitely higher than in the normal lymph gland tissue. A more marked contrast appears when

one compares the lipin content of the walls of the tubercles arising from livers and from lymph glands. The value obtained for the fatty fraction derived from the walls of the lymph gland tubercles is 29.46% of the dry weight; that of the walls of the liver tubercles is only 15.67%, or not much more than half as much. It is worthy of note that the walls of the tubercles from these two sources have, in each case, approximately the same dry weight and ash content, so that the difference in total lipins cannot be explained by the more abundant deposition of inorganic salts in the walls of the liver tubercles. Of the 3 specimens of caseous material, the one coming from the largest liver tubercles contains the highest percentage of fatty material, namely, 18.12% of the dry weight. This slight difference is over-balanced, however, by the fact that the ash content of this caseous material is much lower, indicating a lesser content of inorganic salts.

TABLE 5

RESULTS OF THE ANALYSES OF THE TOTAL LIPIN FRACTIONS OF NORMAL BOVINE LYMPH GLANDS AND LYMPH GLAND TUBERCLES

Source of Tissue	Nature of Specimen	Total Lipins, % of Dry Weight	Cholesterol		Lecithin		Iodin No. after Acid Precipitation	Total N, % of Lipins
			% of Total Lipins	% of Dry Weight	% of Total Lipins	% of Dry Weight		
Peribronchial lymph glands	Normal	22.72	7.93	1.80	36.05	8.49	32.8	0.92
Mesenteric lymph glands	Normal	24.50	5.73	1.40	34.76	8.52	33.9	0.89
Mesenteric lymph glands (long form)	Normal	25.96	5.89	1.53	26.01	6.75	29.9	1.06
Peribronchial lymph glands	Walls of tubercles	29.79	13.52	4.03	25.78	7.68	44.0	1.05
Peribronchial lymph glands	Walls of tubercles	29.73	13.08	3.88	24.98	7.43	45.1	1.12
Peribronchial lymph glands	Walls of tubercles	28.80	13.16	3.84	24.21	7.07	44.0	1.06
Mesenteric lymph glands	Walls of tubercles	29.80	12.30	3.67	26.52	7.98	42.8	0.91
Mesenteric lymph glands	Walls of tubercles	29.19	13.78	3.96	27.55	8.04	41.8	1.09
Mesenteric and peribronchial lymph glands	Caseous material	19.49	26.58	5.18	12.10	2.36	51.2	0.53

As determined by Corper's method, the cholesterol present in the fatty fraction from normal lymph glands represents 6.52% of it, or 1.58% of the dry weight of the specimen. The single specimen of normal peribronchial lymph glands gave a higher cholesterol value than did the specimens of mesenteric glands. From all the specimens of walls of lymph gland tubercles, the amount of cholesterol obtained remained remarkably constant, varying from 12.30-13.78% of the

total lipin fraction. The average for the 5 specimens is 13.17% of the fatty substances, or 3.88% of the dry weight. When compared with the total lipin fraction of normal lymph glands, this percentage is almost exactly twice as much, and at the same time it forms twice as large a percentage of the dry weight, indicating an actual rather than simply a relative increase in cholesterol content. This high cholesterol value for the tubercle walls is explained, at least in part, by the appreciable amounts of caseous material which could not be removed from the fibrous walls and was, therefore, included with them. This caseous material itself contains a much larger percentage of cholesterol than do the walls of the tubercles. In the single specimen here analyzed, the cholesterol constituted 26.58% of the total fatty fraction, or 5.18% of the dry weight. This is over 3 times the amount of cholesterol in normal lymph glands when calculated on the basis of dry weight.

In the estimation of the lecithin in normal bovine lymph glands, the value obtained from the specimen consisting of the long form of mesenteric glands fails to agree with the results obtained with the other specimens, and there is every reason to believe that it is distinctly too low. However, using the average of the 3 determinations, the percentage of lecithin in the total lipin fraction is 32.27, which is equivalent to 7.92% of the dry weight. In the walls of these lymph gland tubercles, the average lecithin value is 25.81% of the fatty fraction, or 7.64% of the dry weight. This is a slight but not significant decrease below that of the normal tissue. The caseous material from these tubercles contained a much smaller percentage of lecithin. It constituted only 12.10% of the total fatty fraction, or 2.36% of the dry weight. Comparing this with the results obtained with the normal tissues, the fatty substances from the caseous material contain only $\frac{3}{8}$ as much lecithin as do the fats from the normal tissues, while on the basis of dry weight they form even a smaller relative fraction. The slight decrease from the normal value noted in the walls of the tubercles may depend in part on the caseous material included in the tubercle walls.

Iodin number determinations were made on portions of the alcoholic solution of the lipins after precipitation of the fats from the water emulsion by means of acid chloroform. While the values given in the table may have some value for the sake of comparison, they do not represent the true iodine numbers of the fats as they occurred in

the normal or in the tuberculous tissues. This is illustrated by the fact that the average of 3 iodine number determinations made on the fats from lymph glands, previous to the acid precipitation, was 41.1, while the same fats after precipitation and re-solution in alcohol gave an average iodine number of 32.2. Before this observation was made, however, all the iodine numbers had been determined on the specimens of tuberculous tissue subsequent to the precipitation of the fats in the acid solution. The iodine numbers obtained for the 5 samples of fats from tubercle walls are fairly constant and are uniformly distinctly higher than those obtained from the normal tissues. The average value is 43.5, as compared with 32.2 for the fats from the normal tissues when similarly treated. This difference is further accentuated when the fats of the caseous material are considered. The iodine number in this case was found to be 51.2.

TABLE 6

THE RESULTS OF THE ANALYSES OF THE TOTAL LIPIN FRACTIONS OF NORMAL BOVINE LIVER AND OF LIVER TUBERCLES

Source of Tissue	Nature of Specimen	Total Lipins, % of Dry Weight	Cholesterol		Lecithin		Iodine No. after Acid Precipitation	Total N, % of Lipins
			% of Total Lipins	% of Dry Weight	% of Total Lipins	% of Dry Weight		
Liver.....	Normal	34.36	2.81	0.97	39.91	13.71	43.4	0.74
Liver.....	Normal	33.38	4.11	1.37	43.00	14.35	38.9	1.07
Liver.....	Normal	34.77	4.31	1.50	40.69	14.15	40.2	0.90
Liver tubercles.....	Walls of tubercles	15.23	15.90	2.42	29.10	4.43	39.8	0.90
Liver tubercles.....	Walls of tubercles	16.11	12.97	2.09	28.33	4.56	40.3	1.16
Liver tubercles.....	Caseous material	17.47	27.20	4.75	16.74	2.91	42.1	1.02
Liver tubercles.....	Caseous material	17.65	26.05	4.61	15.71	2.78	46.0	0.93
Liver tubercles.....	Caseous material	18.12	26.20	4.75	15.45	2.80	42.3	0.88

Total nitrogen determinations were made on the lipin solutions for the purpose of showing how much nitrogen is carried over into this fraction other than that which can be accounted for by the amount of lecithin present. Calculated on the basis of 1 nitrogen atom in a molecule having a molecular weight of approximately 800, the highest amount of lecithin found in any fatty fraction would account for only 0.63% of nitrogen, while the amount actually determined was 0.92% of the total lipins. There is apparently an appreciable amount of nitrogen present in some undetermined form both in the lipins from normal and those from tuberculous tissues.

The cholesterol content of the total lipin fraction of normal bovine liver is here given as 3.74%, when the average of the three values is taken. For comparative purposes, this is probably too low since the first value given in the table is questionable because of failure to get satisfactory separation in the shaking out process.

An additional determination made on the fatty fraction from another normal liver gave the cholesterol content of the fats as 5.07%, or 1.60% of the dry weight. Including this value with those given above, the average becomes 4.07% of the total lipins, or 1.36% of the dry weight. Apparently cholesterol forms a somewhat smaller proportion of the liver lipins than it does of the lymph gland lipins, although on the basis of the dry weights there is no marked difference. The lipins from the walls of the liver tubercles are distinctly rich in cholesterol; it constitutes 14.4% of the fats, or 2.25% of the dry weight. A very much larger percentage of cholesterol, however, occurs in the lipins from the caseous material. For the 3 specimens, the average is 26.48% of the fatty fraction, or over $\frac{1}{4}$ of the entire amount. On the basis of the dry weight, the cholesterol is equal to 4.70%, or 3 times the amount obtained from normal liver. The variations in the lecithin are in the opposite direction as they were also in lymph gland tubercles. In the fats from the normal liver, lecithin constitutes 41.2%, or about 14% of the dry weight. The lipins from the walls of the tubercles contain 28.71% of lecithin, while those from the caseous material contain only 15.9%. Calculated for the dry weights, these values become 4.5% for the fats from the tubercle walls and 2.83% for those of the caseous material. Lecithin is, apparently, only about $\frac{3}{8}$ as abundant in the lipins from the caseous material as in those from normal liver, and it constitutes only $\frac{1}{5}$ as large a fraction of the dry weight.

The iodine number of the fats from normal liver is evidently somewhat higher than that of the fats from normal lymph glands, the average obtained for the liver fats being 40.8 as compared with 32.2 in the fats from lymph glands. For the 5 tuberculous specimens, the iodine number obtained is 42.1, or only a slight increase over that of the fats from normal liver.

The figures obtained for the nitrogen content of the lipins from the liver tissues do not differ in any definite way from those previously given for the lymph gland lipins. There is no apparent tendency for

any larger amount of nitrogen to occur in the lipin fractions from the tuberculous tissues than from the normal tissues, other than that which can be accounted for by the other lecithin percentage.

TABLE 7
RESULTS OF THE ANALYSES OF THE ALCOHOL-ETHER INSOLUBLE FRACTIONS OF NORMAL LYMPH GLANDS AND OF LYMPH GLAND TUBERCLES

Source of Tissue	Nature of Specimen	Alcohol-Ether Residue								
		Alcohol-Ether Residue in % of Dry Wt.	Total N in %	Total N in % of Ash-free Residue	Total P in %	Ash		Calcium		
						% of Alcohol-Ether Residue	% of Dry Wt.	% of Ash	% of Alcohol-Ether Residue	% of Dry Wt.
Peribronchial lymph glands	Normal	76.33	15.56	16.30	1.50	4.56	3.48	11.30	0.52	0.40
Mesenteric lymph glands	Normal	75.03	15.04	15.84	1.58	5.03	3.77	5.78	0.29	0.22
Mesenteric lymph glands (long form)	Normal	75.89	15.93	16.77	1.60	5.03	3.82			
Peribronchial lymph glands	Walls of tubercles	77.08	15.20	16.64	1.80	8.66	6.88	36.88	3.19	2.46
Peribronchial lymph glands	Walls of tubercles	76.18	13.84	15.32	2.38	9.68	7.38	47.35	4.58	3.49
Peribronchial lymph glands	Walls of tubercles	70.25	14.07	15.27	1.87	7.88	5.54	37.80	2.98	2.09
Mesenteric lymph glands	Walls of tubercles	74.44	14.52	15.70	1.83	7.48	5.57	35.20	2.63	1.96
Peribronchial & mesenteric lymph glands	Caseous material	80.80	10.41	14.45	9.88	27.95	22.58	56.27	15.73	12.71

The insoluble residue left after complete extraction of normal lymph glands with alcohol and ether averages in these specimens 75.75% of the dry weight of the tissue. The nitrogen content of this residue is 15.5%; the phosphorus present makes up 1.56% of its weight and the ash constitutes 4.87%. The amount of calcium in the ash is not great enough for accurate determination by the method used, but it constitutes, perhaps, 0.2-0.5% of the alcohol-ether residue. The residues from the walls of the lymph gland tubercles form about the same percentage of the total dry weight as in the normal tissues, the average is 74.5% as compared with 75.75% for normal glands. The percentage of nitrogen in the residues from the tubercle walls is 14.4, while in the normal tissue it is 15.5. This decrease in the percentage of nitrogen is relatively slight when compared with the increase in ash and in calcium. The ash increases from 4.87% in normal tissue to 8.4% in the tubercle walls, while the calcium increases from less than 0.5% to an average of 3.34% of the alcohol-ether residue. The change in the phosphorus content by no means parallels that of the ash.

Its increase is from 1.56-1.97% of the residue. If the amount of calcium found here is combined in the usual way with phosphoric and carbonic acids in the approximate ratio of 4:1, about 1.5% of the phosphorus present would be required to unite with the calcium, leaving only about 0.5% for the organic compounds.

The specimen of caseous material is conspicuous for its high ash and calcium content, in spite of the fact that there were no definitely calcified areas in any of these tubercles. The ash constituted 27.95% of the alcohol-ether residue, or calculated on the basis of the dry weight this is equivalent to 22.58%. The calcium itself made up 15.73%, or approximately $\frac{1}{6}$ of the alcohol-ether residue, while the phosphorus constituted 9.88% of it.

TABLE 8

THE RESULTS OF THE ANALYSES OF THE ALCOHOL-ETHER INSOLUBLE RESIDUES FROM NORMAL LIVER AND FROM LIVER TUBERCLES

Source of Tissue	Nature of Specimen	Alcohol-Ether Residue								
		Alcohol-Ether Residue in % of Dry Wt.	Total N in %	Total N in % of Ash-free Residue	Total P in %	Ash		Calcium		
						% of Alcohol-Ether Residue	% of Dry Wt.	% of Ash	% of Alcohol-Ether Residue	% of Dry Wt.
Liver.....	Normal	68.19	14.95	15.26	0.64	2.03	1.39	19.05	0.39	0.27
Liver.....	Normal	69.25	14.91	15.26	0.69	2.28	1.58	15.80	0.36	0.25
Liver.....	Normal	67.07	15.27	15.51	0.69	2.19	1.47	22.20	0.44	0.30
Liver tubercles	Walls of tubercles	86.94	14.53	15.91	1.82	8.69	7.56	39.45	3.43	2.98
Liver tubercles	Walls of tubercles	88.18	14.61	16.04	1.86	8.90	7.85	43.79	3.90	3.44
Liver tubercles	Caseous material	83.76	10.15	14.35	5.06	29.27	24.52	54.29	15.89	13.31
Liver tubercles	Caseous material	85.49	10.66	14.31	4.82	25.53	21.65	51.07	12.93	11.05
Liver tubercles	Caseous material (large tubercles)	82.82	13.32	16.71	3.59	20.30	16.81	50.50	10.25	8.49

In the 3 specimens of normal liver, the residues left after extraction with alcohol and ether average 68.16% of the dry weight.

Because of the low fat content in the walls of the liver tubercles, an especially high value is obtained for the alcohol-ether insoluble fraction. This forms 87.56% of the dry weight, as compared with 68.16% in the normal tissue. The residues from the caseous material form a slightly smaller percentage of the dry weight, than do the residues from the tubercle walls.

In the normal liver tissue, nitrogen constitutes about 15% of the alcohol-ether residue. Its amount is slightly lower in the walls of the

tubercles where it averages about 14.5%. In the 2 specimens of caseous material from medium-sized liver tubercles, nitrogen forms 10.4% of the residue, although the inorganic materials forming the ash make up 27.4% of this fraction. This value for the nitrogen if calculated on the basis of the organic substances present would form 14.3% of such compounds, which shows that there is no marked reduction in the amount of nitrogen in this caseous material below that which would be present in a corresponding amount of protein under normal conditions. The reduction in the amount of nitrogen is still less in the specimen of caseous material from the large liver tubercles. Here, it constitutes 13.2% of the alcohol-ether residue, although the ash in this case formed $\frac{1}{5}$ of the entire weight of this fraction.

TABLE 9

THE RESULTS OF THE ANALYSES OF THE ALCOHOL-ETHER-WATER INSOLUBLE RESIDUES OF NORMAL LYMPH GLANDS AND LYMPH GLAND TUBERCLES

Source of Tissue	Nature of Specimen	Water Insoluble Residue										
		Water Insol. % of Dry Wt.	Total N in %	Total N in % of Ash-free Residue	Total P		Phospho-protein P, % of P	Ash		Calcium		Purin N % of N
					% of Water Insol.	% of Dry Wt.		% of Water Insol.	% of Dry Wt.	% of Ash	% of Water Insol.	
Peribronchial lymph glands	Normal	67.97	16.12	16.36	0.60	0.41	0.14	1.44	0.98	13.04	0.19	0.32
Mesenteric lymph glands	Normal	69.43	15.52	15.85	0.95	0.66	0.18	2.11	1.46	11.69	0.25	0.57
Mesenteric lymph glands (long form)	Normal	68.79	16.01	16.30	1.02	0.70	0.16	1.78	1.22	6.67	0.12	0.44
Peribronchial lymph glands	Walls of tubercles	72.68	14.46	15.47	1.52	1.10	0.53	6.50	4.72	58.79	3.82	0.21
Peribronchial lymph glands	Walls of tubercles	68.07	14.44	15.80	1.37	0.93	0.65	8.61	5.86	46.08	3.97	0.26
Peribronchial lymph glands	Walls of tubercles	62.61	14.08	15.03	1.16	0.73	0.69	6.30	3.94	43.97	2.77	0.22
Mesenteric lymph glands	Walls of tubercles	63.97	15.65	16.43	1.34	0.86	0.88	4.73	3.03	28.57	1.35	0.28
Peribronchial & mesenteric lymph glands	Caseous material	78.22	10.64	15.81	5.07	3.97	2.04	32.68	25.56	41.13	13.44	0.06

The value obtained for the total phosphorus in the alcohol-ether insoluble fraction of normal liver is 0.67%. In normal lymph glands, the corresponding percentage was 1.56%. In the walls of the liver tubercles, the amount of phosphorus increased to nearly 3 times the amount found in the normal tissue, while the calcium present increased to nearly 10 times that obtained from normal tissue. An exceptionally high phosphorus content occurs in the residues from the caseous material. In these the phosphorus averaged 4.49%, or 7 times that

of normal liver. With this increase in phosphorus, there is much more than a corresponding increase in the amount of ash. For the normal liver, the ash value is about 1.5%, while in the caseous residues, it averages approximately 21%, or 14 times the amount in the normal tissue. The increase in calcium more than parallels the increase in ash; from a normal of 0.4%, it increases to 3.6% in the tubercle walls, and reaches 13% in the caseous residues.

A comparison of the alcohol-ether residues of the specimens of normal lymph glands with the water insoluble fractions makes evident the fact that only a small percentage of the residues goes into solution in water at room temperature. In the water insoluble fraction, there is a decrease of 7.02% of the dry weight below the percentage of the alcohol-ether residue. In the specimens from the walls of the lymph gland tubercles, the decrease is 7.65%, and for the caseous material 2.58%. The water soluble materials form, apparently, a smaller percentage of the caseous substances than they do of the normal tissues or of the tubercle walls. In nearly every case, the total nitrogen present in the water insoluble residues constitutes a slightly higher percentage than it does of the alcohol-ether residues. The loss in weight is evidently due to the solution of substances relatively poorer in nitrogen than those which remain. As a result of the extraction with water, the residues suffer a loss in their phosphorus content. This is a decrease of approximately 45% in the normal tissues and about 40% in the tuberculous specimens. That portion of the phosphorus which is split off from the residues by 1% NaOH is here listed as phosphoprotein phosphorus. In the normal tissues this constitutes about $\frac{1}{5}$ of the total phosphorus, while in the tuberculous specimens it forms a much larger part of the total phosphorus, sometimes even more than half. These high values are, doubtlessly, due to a solution of a part of the inorganic phosphorus when the alkaline solution is neutralized with acetic acid. The percentages of ash and calcium are usually somewhat lower in the water insoluble fractions than in the alcohol-ether residues. The determinations of purin nitrogen in the water insoluble residues of normal lymph glands gave an average of 0.44% of these residues, while there was a distinctly smaller amount in the tubercle walls where the percentage was 0.24%. The single specimen of caseous material from lymph glands seemed to contain only a trace of purin nitrogen, the value obtained being 0.06% of the water insoluble fraction. This low content of purin nitrogen in

caseous material is not surprising, but it stands out in striking contrast to the exceptionally high percentages obtained from the caseous material from liver tubercles.

TABLE 10

THE RESULTS OF THE ANALYSES OF THE WATER-INSOLUBLE RESIDUES FROM SPECIMENS OF NORMAL LIVER AND FROM LIVER TUBERCLES

Source of Tissue	Nature of Specimen	Water Insoluble Residue										
		Water Insol. % of Dry Wt.	Total N in %	Total N in % of Ash-free Residue	Total P		Phospho-protein P, % of P	Ash		Calcium		Purin N % of N
					% of Water Insol.	% of Dry Wt.		% of Water Insol.	% of Dry Wt.	% of Ash	% of Water Insol.	
Liver.....	Normal	65.91	15.20	15.26	0.25	0.17	0.09	0.39	0.26			0.15
Liver.....	Normal	65.48	15.12	15.17	0.27	0.18	0.06	0.34	0.22			0.14
Liver.....	Normal	64.17	15.22	15.39	0.24	0.16	0.06	1.08	0.71	55.56	0.60	0.10
												0.11
Liver tubercles	Walls of tubercles	81.52	13.99	15.56	2.26	1.84	1.03	10.08	8.22	54.39	5.49	0.16
Liver tubercles	Walls of tubercles	81.18	14.17	15.43	1.51	1.23	0.83	8.19	6.65	49.49	4.05	0.16
												0.14
Liver tubercles	Caseous material	76.63	10.64	15.14	5.34	4.09	2.34	29.75	22.80	59.08	17.58	0.26
Liver tubercles	Caseous material	80.14	10.97	15.07	4.57	3.66	2.39	27.21	21.81	61.71	16.79	0.27
Liver tubercles	Caseous material (large tubercles)	79.66	13.05	15.48	3.58	2.85	2.15	15.69	12.50	40.15	6.30	0.27

In the specimens of normal bovine liver, the water insoluble fractions form 65.18% of the dry weight, while the alcohol-ether residues constitute 68.17%. As a result of the extraction of these residues with water, the amount which goes into solution, together with a small mechanical loss, is 3% of the dry weight of the specimen.

From the residues of the tubercle walls, 6.21% of the dry weight passed over into the water soluble fraction, and from the residues of the caseous material, 5.21%. Here, as in the lymph gland tissues, the percentage of nitrogen in the water insoluble residues is slightly higher than in the alcohol-ether residues. In the normal specimens the total phosphorus is reduced to less than half that of the alcohol-ether residues, but there is no corresponding reduction in the phosphorus in the specimens from tuberculous tissues; in these, the phosphorus values remain practically unchanged. The amount of ash, likewise, is decreased in the normal specimens, but remains nearly constant in the tuberculous residues. Calcium, as a rule, forms a larger percentage of the water insoluble fraction than it does of the alcohol-ether residues. The average value obtained for the purin

nitrogen in the residues of normal liver is 0.13% of the water insoluble fraction; in the residues from the tubercle walls, a similar percentage is obtained, namely, 0.15. Very strangely, three closely agreeing determinations of purin nitrogen made on the residues of caseous material from liver tubercles gave a distinctly higher purin content than that of normal liver or of the liver tubercle walls. Here, the percentage obtained was 0.27, as compared with 0.13 for normal liver and 0.15 for the tubercle walls.

TABLE 11

THE RESULTS OF THE ANALYSES OF THE WATER SOLUBLE FRACTION OF NORMAL BOVINE GLANDS AND LYMPH GLAND TUBERCLES

Source of Tissue	Nature of Specimen	Water Soluble Fraction						
		Nitrogen					Phosphorus	
		Total N in % of Dry Weight	Pro- teose N in % of Total N	Am- monia N in % of Total N	Free Amino- Acid N in % of Total N	Amino- Acid N in % of Total N	Total P in % of Dry Weight	Inor- ganic P in % of Total P
Peribronchial lymph glands	Normal	1.59	7.81	7.38	21.10	27.59	0.88	54.54
Mesenteric lymph glands	Normal	1.28	9.03	1.00	23.59	36.48	0.74	56.20
Mesenteric lymph glands	Normal	1.41	9.37	Trace	14.33	30.85	0.66	50.21
Peribronchial lymph glands	Walls of tubercles	0.89	16.90	7.48	21.40	28.33	0.50	78.83
Peribronchial lymph glands	Walls of tubercles	0.88	12.90	5.16	15.80	33.12	0.53	88.26
Peribronchial lymph glands	Walls of tubercles	0.83	11.65	2.22	28.68	38.20	0.48	85.89
Mesenteric lymph glands	Walls of tubercles	1.66	24.58	7.80	18.61	36.40	0.59	80.21
Peribronchial and mesenteric lymph glands	Caseous material	0.26	35.20	Lost	14.89	26.80	0.20	93.50

The total nitrogen in the water soluble fraction from the specimens of normal lymph glands is equivalent to 1.43% of the dry weight of the specimens. The percentage of nitrogen in the corresponding fractions from the walls of the peribronchial lymph gland tubercles is, in each case, definitely lower than in the normal tissues, the average being 0.87% instead of 1.43%. The tubercle walls from the mesenteric lymph gland tubercles appear, from Table 11, to be exceptional in their high content of water soluble nitrogen, but this is explained by the fact that a clear solution was not obtained by centrifuging and some protein material was carried over in suspension.

The amount of water soluble nitrogen in the specimen of caseous material is exceptionally low, constituting only 0.26% of the dry

weight, or less than $\frac{1}{3}$ of that derived from the normal tissue. There seems to be a rather definite increase in the proteose nitrogen in the tuberculous as compared with the normal tissues, the caseous material being the richest in this form of nitrogen compounds. The values obtained for ammonia nitrogen are quite inconstant, as are those, also, for the free amino-acids. The attempt to determine a peptone nitrogen fraction, following acid hydrolysis of samples of the water soluble substances, was eminently unsatisfactory. The value obtained for the peptone nitrogen was often a negative one. A slightly smaller percentage of phosphorus, when calculated on the basis of the dry weight, goes into the water solution from the tuberculous than from the normal tissues. This difference is most marked in the specimen which was completely caseous. On the other hand, of the phosphorus which does enter the water fraction, an increasing large percentage of it is inorganic phosphorus in the specimens of tuberculous tissues.

TABLE 12

THE RESULTS OF THE ANALYSES OF THE WATER SOLUBLE FRACTIONS OF NORMAL BOVINE LIVER AND OF LIVER TUBERCLES

Source of Tissue	Nature of Specimen	Water Soluble Fraction						
		Nitrogen					Phosphorus	
		Total N in % of Dry Weight	Pro- teose N in % of Total N	Am- monia N in % of Total N	Free Amino- Acid N in % of Total N	Amino- Acid N in % of Total N	Total P in % of Dry Weight	Inor- ganic P in % of Total P
Liver.....	Normal	0.75	5.44	2.43	18.35	32.20	0.49	66.25
Liver.....	Normal	0.81	2.11	7.11	11.50	23.84	0.56	58.91
Liver.....	Normal	0.96	4.27	0.42	9.21	29.49	0.59	57.61
Liver tubercles.....	Walls of tubercles	0.68	39.19	5.10	16.10	41.45	0.26	77.58
Liver tubercles.....	Walls of tubercles	0.71	37.45	5.42	18.23	34.80	Lost	Lost
Liver tubercles.....	Caseous material	0.24	13.37	10.72	37.46	40.05	0.17	84.51
Liver tubercles.....	Caseous material	0.23	26.12	9.87	38.38	43.30	0.14	Lost
Liver tubercles.....	Caseous material (Large tubercles)	0.29	14.01	Lost	23.57	27.87	0.21	80.88

In liver, as well as in lymph gland specimens, the higher percentage of water soluble nitrogen is obtained with normal rather than with tuberculous specimens. The difference, in this respect, between normal liver and the walls of liver tubercles is slight, but only about $\frac{1}{3}$ as much nitrogen goes into solution from the caseous material. The ammonia nitrogen fraction is apparently somewhat increased in the

caseous material from liver tubercles, as is also the free amino-acid content. The value obtained for peptone nitrogen is negative in every specimen of tuberculous liver, although it has a positive value in the normal tissues. The phosphorus entering the water solution from the tuberculous tissues, in every case, constitutes a smaller percentage of the dry weight than with normal tissues.

Here, again, an increasingly large percentage of the total phosphorus is inorganic phosphorus in the water solutions from the tuberculous tissues.

THE ANALYSIS OF CASEOUS MATERIAL FROM HUMAN LYMPH GLAND TUBERCLES

This specimen consisted of the caseous material from 3 tracheo-bronchial lymph glands. The largest of these glands was about 3.5 cm. in its greatest dimension and it was completely caseous without any definite areas of calcification in it, other than fine sandlike particles. The two smaller glands were caseous and partly calcified. The entire specimen weighed only 13.5 gm. and was, therefore, too small for accurate analysis.

In spite of the fact that most of the specimen formed a semifluid mass, its dry weight was 60.7% of the moist weight, or the water present formed only 39.3% of the original weight. From the entire specimen 0.45 gm. of lipins was obtained, which is equivalent to 5.5% of the dry weight, or 19.7% of the ash-free residue. Unfortunately, the cholesterol was lost. Lecithin was found to constitute 30.9% of the total lipins, a percentage $2\frac{1}{2}$ times as great as that of the fats from bovine caseous material. This lecithin value represents 1.7% of the dry weight, or 6.1% when calculated on the ash-free basis.

The iodine number of these fats was found to be 30.7, which is about the same as that obtained for the fats from normal bovine glands, but much lower than that from the single specimen of caseous material.

The alcohol-ether residue of this specimen formed 93% of the dry weight, and the water-insoluble fraction 88.9%. The total nitrogen determinations made on the alcohol-ether residue and on the water-insoluble fraction gave 2.46 and 2.33% of nitrogen, respectively. When the ash content of these residues has been deducted, these nitrogen values become 10.93 and 10.84%. This represents a reduc-

tion of the organic substances far below that seen in any of the specimens of caseous material from bovine tissues. The total phosphorus content of each of these residues was 9.25-9.50%, while the ash constituted 77.5-78.5%. Of this ash the calcium formed over 60% of its weight. An attempt was made to evaluate the purin nitrogen, but evidence of only a trace of purins was obtained.

DISCUSSION OF RESULTS

The results of the analyses of normal bovine lymph glands agree closely, so far as they are comparable, with those obtained by Bang.⁷ For the water content and dry weight of mesenteric glands of oxen, he reported 80.41% of water and 19.59% of solids, as compared with the figures here given of 81.59% of water and 18.41% of solids for glands having the same origin. There is a similar close agreement on the percentage of fatty substances present. Bang gave the alcohol soluble substances as 4.76% of the fresh weight; they are here reported as constituting 4.49%, or the equivalent of 24.39% of the dry weight. The percentage of ash given by Bang is 1.05%, a value somewhat higher than that obtained in these analyses. The water content of normal bovine liver as given by v. Bibra¹ is 71.39% of the moist weight and the average of Oidtmann's determinations is 71.66%. For the two livers examined in these analyses, the water content was found to be 70.63%, leaving a dry weight of 29.37%. No such close agreement exists with regard to the fat content of bovine livers. v. Bibra gives percentages of 2.64 and 3.28, based on the fresh weight, or when calculated on the dry weight, the average is 10.35. Profitlich found the fat to vary from 10.87-21.78% of the dry weight. In the two normal livers which I have examined, the total lipins constituted 31.28 and 34.17% of the dry weight, or an average of 32.72%. There was no macroscopic evidence of pathologic fatty changes in either of these livers and the higher percentages obtained are probably due to the method of extraction.

In the tuberculous tissues, the finding of a higher fat content in the walls of lymph gland tubercles than in the completely caseous material agrees with the observations made by Wells in regard to the scrapings from tubercle walls, but differs in the fact, that in this caseous material both calcium and phosphorus were present in much larger amounts than in the tubercle walls, so that a part of the decrease in total lipins may be attributed to the deposition of calcium salts.

His finding that the water soluble fraction of the caseous liquid content of tubercles constitutes a smaller percentage of the dry weight than it does in the scrapings from the walls is confirmed by these analyses. A similar low lipin content was found for the caseous material from human lymph glands, but here there is a correspondingly great increase in inorganic salts and a like decrease in the protein constituents. Bossart obtained about the same amount of fat from pure caseous material from human lymph glands as was obtained here for the caseous material of bovine origin. The values which he reports for his partially caseous specimens are lower, however, than those reported here for the walls of tubercles or for normal lymph gland tissue. According to Bossart's analyses, cholesterol apparently made up a larger percentage of the total fats in the partially caseous material than it did in the completely caseous specimen. In bovine lymph glands and livers, cholesterol seems to constitute a much larger percentage of the fats from the caseous material than it does of the fats from the walls of tubercles or from normal tissues.

The variation in the amount of lecithin is in the opposite direction. It is more abundant in the specimens of normal tissue than it is in the tuberculous ones and the amount in the caseous material constitutes the smallest percentage of the total fats, as well as of the dry weight. This finding of a decrease in the lecithin content of the fats from the caseous material harmonizes with the similar finding by Wells in his study of the fats of livers in acute yellow atrophy and in delayed chloroform poisoning, and also with the observation made by Griniew on the organs of tuberculous guinea-pigs.

So far as lymph gland tubercles are concerned, the results obtained by these analyses seem to support the evidence furnished by staining methods that the walls of tubercles contain a larger amount of fat than does the caseous material itself. While this does not hold true of liver tubercles, it seems quite likely that the difference is due to the more rapid formation of the tubercles in the liver tissue which is already extremely rich in fats. In all 4 specimens of caseous material, the total lipins constitute a smaller percentage of the dry weight than in the normal tissues from which this caseous material originated. This shows conclusively that caseous material is not so rich in fats as it has usually been considered.

The cholesterol of the total lipins increases at about the same rate that the lecithin decreases in the tuberculous tissues, so that the sum

of the 2 percentages remains practically constant, leaving the simple fats to form about the same percentage of the total lipins in normal and tuberculous tissues.

In the alcohol-ether-insoluble residues, the percentage of total nitrogen is slightly higher in the normal lymph gland residues than in those from normal liver, in spite of the fact that ash content of the lymph gland residues averages twice that of the normal liver residues. The walls of the tubercles arising from these 2 tissues give residues which agree more closely in their nitrogen content than do the tissues from which they arise, just as they also resemble each other more closely in their histologic structure. The residues from 3 of the specimens of caseous material give approximately the same percentage of nitrogen, and about the same percentage of ash, whether from lymph gland or from liver tubercles. In the residues from the caseous material of the large liver tubercles, the higher content of nitrogen is dependent in part on the smaller amount of inorganic salts present, and probably in part also on the more rapid necrosis than that which occurs in the formation of the smaller tubercles, so that less extensive changes have taken place in the proteins originally present in the area.

The total phosphorus content of the normal lymph gland residues averages twice that of the residues of the normal liver. This can be explained by the greater amount of nucleoproteins in the lymph glands. The walls of the tubercles arising in lymph glands or in bovine liver give residues which contain approximately the same amount of phosphorus. As compared with the phosphorus content of the normal tissues, the increase in the amount of phosphorus in the walls of the lymph gland tubercles is small as compared with the increase in the ash content. This is apparently due to a decrease in the nucleoproteins and their replacement by proteins poorer in phosphorus, together with the deposition of inorganic salts. In the residues from liver tubercles, the amount of phosphorus is increased to nearly 3 times the amount in normal tissue, although the total ash content is only slightly higher than that of the walls of lymph gland tubercles. In this case there was no tissue rich in nucleins to be replaced, so that the increase in the phosphorus is due chiefly to the deposition of inorganic salts.

In the water-insoluble residues of lymph glands and lymph gland tubercles, the purin nitrogen decreases with the tubercle formation

and reaches a minimum in the residues of caseous material. As lymph gland tissue is replaced by fibrous tissue relatively poor in nuclein substances, a decrease in purin nitrogen would be expected in the tubercle walls. Likewise, in caseation, as the nuclear substances disappear, as shown by staining methods, a further reduction of purin content probably also occurs. From the results obtained with residues from normal liver and from liver tubercles, the tubercle walls are apparently slightly richer in nucleoproteins than is the normal liver. A finding which, at present, cannot be explained is the distinctly greater purin content of the caseous residues of liver tubercles as compared with the purins in the residues of normal liver and in walls of liver tubercles. Three closely agreeing determinations give an average value $\frac{1}{2}$ more than that of the tubercle walls and approximately twice that of normal liver tissue. This does not conform with the finding of an extremely low percentage of purin nitrogen in the single specimen of caseous material from bovine lymph gland tubercles.

SUMMARY

The water content of normal bovine lymph glands constitutes about 81 or 82% of the moist weight. No very distinct differences are noted between peribronchial glands and those from the mesenteric region. The tubercle walls and the caseous material from lymph gland tubercles contain a lower percentage of water than does the normal tissue.

In normal bovine liver tissue, the percentage of water present is less than that of the tubercle walls or of the caseous material from liver tubercles. The specimens of caseous material from lymph gland and liver tubercles approach each other closely in their water content, the average being about 75% for the bovine material.

The alcohol-ether-soluble substances from normal bovine lymph glands form about 24.4% of the dry weight, or about 4.4% of the moist weight. The walls of the lymph gland tubercles contain a distinctly larger amount of lipins than does the caseous material or the normal tissue. On the contrary, the walls of liver tubercles are poor in lipins as compared with the normal tissue, and they contain a smaller amount of fats than does the caseous material from these tubercles. When calculated on the basis of the dry weight, the caseous material from lymph gland tubercles contains a smaller percentage of lipins than does normal lymph gland tissue. When the ash is deducted, this difference disappears and the content of lipins becomes

equal to or slightly greater than that of the normal tissue, but less than that of the tubercle walls. When calculated on an ash-free basis, the lipin content of the caseous material from liver tubercles is distinctly less than that of the normal tissue but greater than the lipin content of the tubercle walls.

Cholesterol forms about 6.5% of the lipins from normal bovine lymph glands, or about 1.5% of the dry weight. The lipins from the walls of lymph gland and liver tubercles contain, in every case, 2-3 times as much cholesterol as do the lipins from the normal tissues. This is an actual increase also when calculated on the basis of the dry weight. The caseous material contains even a larger percentage of cholesterol than do the tubercle walls.

Lecithin constitutes about 32% of the lipin fraction of normal bovine lymph glands, or about 7.9% of the dry weight; the corresponding values for normal liver are 41.2% of the fats, or 14% of the dry weight. The lecithin content of the fats from the tubercle walls is slightly less than that of the normal tissues, while there is a very marked reduction in the lecithin content of the lipins from caseous material of bovine origin. In the specimen of caseous material from human lymph glands, lecithin formed 30.9% of the total lipins.

The iodine numbers obtained for the fats of the tuberculous specimens from lymph glands are higher than those from the normal tissues. This observation does not hold true for the liver specimens. In the latter, there is no difference noted between the iodine numbers obtained for the lipins from normal and tuberculous specimens, although the values are practically the same as those from the fats from the lymph gland tubercles.

In the residues of caseous material left after extraction with alcohol and ether, the nitrogen content remains relatively high, in fact, the reduction in nitrogen content is only slight when the calculations are made on ash-free residues. The percentage of nitrogen does not differ much from that obtained from the normal proteins of these tissues.

In specimens of caseous material in which there are no macroscopic evidences of calcification other than the presence of sandlike particles, calcium sometimes forms as much as 15% of the residue left after extraction of the fats. In such residues, the phosphorus content may reach 9%.

The amount of purin nitrogen in the walls of lymph gland tubercles is only slightly more than half that of normal lymph gland tissue, and the amount is apparently much less in the caseous material. In the residues from the walls of liver tubercles, purin nitrogen is present in only slightly higher percentage than in the normal liver. The results here obtained would seem to indicate that the purins are even more abundant in the caseous residues of liver tubercles.

The amount of material which enters the water solution during extraction is distinctly less from caseous material than from the residues of normal tissues.

A STUDY OF THE GRAM-NEGATIVE BACILLI OF RENAL INFECTIONS

RUSSELL D. HERROLD AND HARRY CULVER

From The John McCormick Institute for Infectious Diseases, the Department of Pathology and Bacteriology, University of Illinois College of Medicine, and the Cystoscopic Department of Cook County Hospital,

CHICAGO

In a recent report on the bacteria found in pyogenic renal infections we¹ stated that 85% of 116 patients were infected with gram-negative colonlike organisms either in pure or mixed form. No attempt was made at that time to classify these organisms other than to determine that they belonged to the colon or closely allied groups.

Clinical observation indicates that there must be either a vast difference in the virulence of the various strains in the colon group or an equally marked difference in the susceptibility of individual patients. The contrast in the lesions produced and course of the infection between the staphylococcus infections and those of the colon group as a whole is well known. It may be that equally prominent and important differences exist between the infections produced by the various colon groups.

The purpose of this paper is a classification of 86 different strains of colon bacillus-like organisms by means of cultural, fermentative and serologic methods. These organisms were all secured by ureteral catheter from the kidneys and isolated by cultivation on blood-agar plates.

It is a mere coincidence that just half of the organisms studied belong to the true colon group while the other half does not comply with the described characteristics of true colon organisms, but approach very closely the paracolon organism described by Mackie.² The essential difference lies in the fact that this last group does not ferment lactose and alkalizes rather than acidifies milk.

The motility tests were made on hanging drops of suspensions in broth taken from 18-24 hour old cultures. The indol determinations

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¹ Jour. Am. Med. Assn., 1918, 70, p. 1444.

² Jour. Path. and Bacteriol., 1913, 18, p. 137.

were made on 4-day old cultures in sugar-free broth using the para-dimethylamid-benzaldehyd method, while the reactions on gelatin were read after 6 weeks' cultivation at room temperature.

The various sugars were made in 1% strength in Hiss serum water medium. Following inoculation the reactions were read after 48 hours' incubation at 37 C.

TABLE 1
CULTURAL CHARACTERISTICS OF GRAM-NEGATIVE BACILLI FROM CATHETERIZED SPECIMENS OF URINE FROM THE KIDNEY

Groups With Number of Strains		Motility	Litmus Milk	Gelatin	Indol Reaction	Dextrose	Lactose	Mannose	Mannite	Saccharose	Dulcitate	Raffinose	Morphology
Paracolon Type 43 Strains		+	—	—	—	+	—	+	*	—	—	—	Gram-negative, pleomorphic bacilli, with tendency to form vacuoles, especially after 24 hours old.
True Colon Type 43 Strains	A 15	—	+	—	+14 —1	+	+		*	—	+		Typical gram-negative short plump bacilli.
	B 12	+2 —10	+	+1 —11	+	+	+		*	+	+		
	C 9	+1 —8	+	+1 —8	+	+	+		*	—	—		
	D 7	+2 —5	+	—	+5 —2	+	+		*	+	—		

* Variable.

Organisms are classified in the true colon group when they acidify litmus milk, ferment dextrose, lactose and mannite, produce indol and have no action on gelatin. The motility of organisms of this group is seen to be variable while all other characteristics conform very closely to type, there being 2 strains that partially liquefied gelatin, but are so similar to the others in all other respects that they are classified as true colon bacilli and not as members of the proteus group. Attention is directed to the fact that not one of these 86 organisms is classified as a proteus bacillus, while the older literature and text books speak of proteus bacilli and colon bacilli with the same degree of emphasis, and as being common infecting organisms of the urinary tract.

The fermentative reactions vary markedly among members of the true colon group. This is brought out in Table 1, showing that even those having similar fermentative reactions may have different characteristics in other respects; however, such irregularities are seen to be uncommon.

For the sake of emphasis the true colon organisms are divided according to their fermentative ability into 4 groups as seen in Table 2.

TABLE 2
FORTY-THREE TRUE COLON ORGANISMS CLASSIFIED ACCORDING TO FERMENTATION REACTIONS

	Dextrose	Saccha- rose	Lactose	Mannite	Dulcite	Number of Strains
<i>B. Coli Communis</i>	+	—	+	+	+	15 A.
<i>B. Coli-Communior</i>	+	+	+	+	+	12 B.
<i>B. Acidi-lactici</i>	+	—	+	+	—	9 C.
<i>B. Aerogenes</i>	+	+	+	+	—	7 D.

Columns marked A., B., C., and D. same as in Table 1.

The immunity reactions of bacilli belonging to the colon group show a striking contrast to the action of immune serum for related organisms as *B. typhosus* and *B. paratyphosus*. A serum immune to a particular strain of *B. typhosus* will agglutinate the great majority of all other typhoid strains with small variations in degree. On the other hand, serum immune to various *B. coli* groups have been found to exert little or no action on strains which correspond in fermentative reactions and other characteristics to those used for immunization. It has even been considered that the action of colon agglutinin is practically limited to the particular strains used for immunization.

Immune serum was obtained for 4 strains belonging to the para-colon and a like number for true colon organisms. The technic for obtaining immune serum is as follows:

Half-grown rabbits were injected intravenously with killed cultures of bacilli in salt solution in 2 series thus: First day 1/100, 2nd day 1/75, 3rd day 1/50 of a 24-hour growth of an agar slant in 2 cc suspension; 9th day 1/50, 10th day 1/25, and 11th day 1/10 of the same growth. On the 15th day the rabbits were killed and the blood collected. The serum was removed and placed in sterile bottles without preservative. In all 8 serums there was a specific agglutinin titer of 1:1,280 or higher which seemed sufficient to distinguish group agglutinins if any were present.

Dilutions of the serum to be tested were made in small tubes with salt solution; a salt solution control was used. Thick suspensions were made of the 24-hour growth of bacilli from plain agar. An amount of this suspension equal to the amount of fluid already in each tube was added, making the serum dilutions range from 1:20 to 1:1,280. The tubes were incubated for 3 hours at 37 C. and then allowed to stand at room temperature for 24 hours before the readings were made.

As previously stated the specific agglutinin titer for each antiserum was 1:1,280 or more. To ascertain whether or not nonspecific agglutinin was present, cross agglutination tests were made, using the 8

antisera and testing the activity of each against the 8 strains used for immunization.

Table 3 represents results of the cross agglutination experiments, using 4 antiparacolon serums and 4 anticoli.

TABLE 3
CROSS AGGLUTINATION WITH EIGHT MONOVALENT SERUMS OF HIGH TITER

Number of Strain and Group	Antiparacolon Serums				Anticolon Serums			
	285	170	277	271	143	211	257	281
Paracolon 285.....	1,280*	1,280	1,280	1,280	320	40	0	40
Paracolon 170.....	1,280	1,280	1,280	1,280	40	80	0	0
Paracolon 277.....	1,280	1,280	1,280	1,280	0	0	0	0
Paracolon 271.....	1,280	40	1,280	1,280	40	40	40	0
Colon 143.....	80	40	0	0	1,280	640	0	0
Colon 211.....	40	20	0	20	320	1,280	80	0
Colon 257.....	160	40	0	80	0	40	1,280	20
Colon 281.....	40	40	40	40	0	0	0	1,280

* The figures placed in columns under each antiserum express the highest dilution of that serum which will agglutinate the organism represented by the numbers in the first left hand column.

It is seen that any antiparacolon serum agglutinated all the paracolon organisms tested as readily as the organism used to produce the serum, each organism being agglutinated at 1 to 1,280 by each of the 4 antisera with one exception. This result is in accord with the usual statement that members of this particular group of organisms are closely related as far as their reactions to immune serum is concerned. In this respect they simulate *B. typhosus* and *B. paratyphosus*. The same antiparacolon serums have only little or no agglutinating power for members of the colon group. This condition varies for each colon strain, some strains being agglutinated in dilutions of 1:160, while there are other strains that appear not to be affected by the action of any of the antiparacolon serums.

In marked contrast to the action of antiparacolon serum is that of anticolon serum which has a quantitatively specific action. Whereas each serum agglutinates the homologous organism in dilutions of 1:1,280 in each instance, it has very little or no agglutinating power for the other organisms of the colon group. The highest nonspecific agglutination is 1:320, while in most instances no agglutination is shown whatsoever. The anticolon serums react against the paracolon organisms very weakly and irregularly, their action being not unlike the action of paracolon serum on colon organisms.

To determine whether all of the paracolon organisms are biologically closely related, as indicated in Table 3, it seemed pertinent to

investigate further the agglutinating activity of certain antiparacolon serums against a greater number of paracolon strains, thus tending to minimize the possibilities of coincidence. Therefore 2 antiparacolon serums were tested against the entire 42 paracolon strains. The result is given in Table 4.

TABLE 4
AGGLUTINATION OF PARACOLON ORGANISMS BY TWO MONOVALENT SERUMS OF SAME GROUP

Antiparacolon Serum	Serum Dilution	Number of Strains
No 285.....	1,280	18
No 170.....	1,280	
No 285.....	640	4
No. 170.....	80	
No. 285.....	80	4
No. 170.....	640	
No. 285.....	160	17
No. 170.....	160	

The uniform response of members of this group to agglutination is not so pronounced as might be concluded from a less extensive investigation. From Table 3 it may be concluded that these organisms are very closely related in every way, while a more comprehensive analysis justifies the belief that this is also a heterogeneous group, as only 18 of the 43 strains tested reacted like the organism used to produce the immune serum; that is, agglutinated at a dilution of 1:1,280. The remaining 25 strains can be divided serologically into 3 groups as seen in Table 4. While the group is quantitatively heterogeneous it is noteworthy that the members are not unrelated, as in most instances some agglutination takes place in all strains tested.

It becomes apparent that all members of both the colon and paracolon groups are liable to vary in one or many characteristics, thus any 2 strains may be different biologically, but in other respects very closely related. With these facts in mind there would appear to be no grounds for vaccine therapy in these infections unless the specific infecting organism were used for immunizing purposes.

An analysis of the clinical phase of these infections fails to reveal any difference either in the symptomatology, the changes or the course of the infection produced by any distinct subdivision of this large group.

Of the two most chronic infections encountered, in neither one of which we were able to secure a bacteriologic cure, one was due to a member of *B. coli-communis* group and the other to a paracolon

bacillus. All other bacilli tested were influenced by treatment regardless of the type of infection. In the two infections mentioned it seemed that local renal conditions might have been responsible for the chronicity of the course, as one patient had a moderate degree of hydronephrosis and the other had passed a renal stone.

There appears to be no symptoms peculiar to any type of infection and there is no apparent disparity in the severity of the infections with reference to distinct groups. Therefore, from these observations it may be concluded that the chief factors to determine the course of an infection by the heterogeneous *B. coli* group lie in the local and general conditions of the patients and not in the type of bacillus producing the infection. More detailed and extended observations are, of course, necessary to substantiate the foregoing assertion.

CONCLUSIONS

In a series of 86 strains of gram-negative bacilli from renal infections but one-half were found to be true colon bacilli, the other half being paracolon organisms.

The true colon bacilli were found, by means of fermentative reactions, to be members of four distinct groups. This grouping bears no relation to specific antibody reaction, since an antiserum for any one organism does not react with equal vigor against other members of the same or different groups.

By means of agglutination tests it was demonstrated that the colon group is markedly heterogeneous, as a monovalent serum reacts almost specifically. The paracolon group, however, while more closely related serologically than the members of the colon group, is still a heterogeneous group, but many members of this group reacted exactly alike in every respect in which they were tested.

There is no apparent clinical similarity between any two infections by members of any one group or subgroup. The difference between the various infections seems to be due to local or general conditions of the patient and not to any particular gram-negative bacillus.

A STUDY OF COMPLEMENTS USED IN THE WASSERMANN REACTION

E. H. RUEDIGER

From the Pathological Laboratory of the Bismarck Hospital, Bismarck, N. D.

The serums commonly used as complement in the Wassermann reaction are those of guinea-pig and of man. That the complement content of serums varies has repeatedly been shown and Browning and McKenzie¹ found that complements vary in fixability.

Complements usually deteriorate rapidly and several workers sought by artificial means to prevent such deterioration. Noguchi dried it on filter paper, others kept it in a frozen state and more recently Ramy² proposed sodium acetate as a preservative for complement.

The use of human complement has been advocated by Hecht,³ Tschernogubow,⁴ Fleming,⁵ Emery,⁶ von Dungern,⁷ Gurd,⁸ Noguchi⁹ and others.

In this report the following points are considered:

1. Fixability and complement content of fresh guinea-pig serum.
2. Fixability and complement content of fresh human serum.
3. Noguchi's recent method compared with our regular method.
4. The effect of normal human serum on syphilitic human serum.
5. The effect of normal guinea-pig serum on syphilitic human serum.
6. The rate at which syphilitic serum is altered by normal serum.
7. Deterioration of guinea-pig complement in the refrigerator.
8. Sodium acetate as a preservative for complement.
9. Glycerol as a preservative for complement.

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¹ Ztschr. f. Immunitätsforsch. u. exper. Therap. 1909, 2, p. 459.

² Jour. Am. Med. Assn., 1917, 69, p. 973.

³ Wiener klin. Wchnschr., 1909, 22, p. 338.

⁴ Deutsch. med. Wchnschr., 1909, 35, p. 668.

⁵ Lancet, 1909, 1, p. 1512.

⁶ Lancet, London, 1910, 179, p. 732.

⁷ Münch. med. Wchnschr., 1910, 57, p. 507.

⁸ Jour. Infect. Dis., 1911, 8, p. 427.

⁹ Jour. Am. Med. Assn., 1918, 70, p. 1157.

METHOD

In our regular method the serum to be tested is heated to about 55.5 C. for 30 minutes and is mixed with an equal volume of sterilized glycerol. Six test tubes are used and each receives 0.2 cc of serum-glycerol mixture. Complement is used in quantities of 0.2 cc of a 1:5, 1:10 and 1:20 dilution. Of antigen, alcoholic extract of human heart muscle, the largest dose that is no longer anticomplementary, is used and is diluted so that the test dose is contained in 0.2 cc. Human corpuscles are well washed, the washed blood corpuscles are made up into a 2.5% suspension in salt solution (0.9%), and the test dose is contained in 0.2 cc of the suspension. Hemolytic amboceptor is prepared in rabbits and is used in doses of 1 unit per test tube. The term unit is applied to the smallest quantity which with 0.2 cc of 1:10 dilution of complement in the presence of 0.2 cc of serum-glycerol mixture completely dissolves the test dose of blood corpuscles in one hour. The total quantity in each test tube is 1 cc, first incubation is in the refrigerator for 5 hours and second incubation in the incubator for one hour. After the tubes have stood at room temperature for from 1-2 hours the results are read and recorded.

TEST 1

Ten guinea-pig serums, Serums 1-10, inclusive, were tested on 12 syphilitic human serums. All complement serums were used singly and in mixtures of 5 serums each. The quantity of amboceptor was kept uniform throughout.

TABLE 1
COMPARISON OF TEN GUINEA-PIG COMPLEMENTS

Number of Serum	Number of Complement	Sex of Guinea-Pig	Amboceptor per Tube Unit	Readings*						Results
				Antigen Tubes			Control Tubes			
				1	2	3	1'	2'	3'	
1	1	M	1 g	+	0	0	+	+	±	Strongly positive, 5+.
	2	F	1 g	+	tr	0	+	+	±	Strongly positive, 4+.
	3	M	1 g	+	0	0	+	+	tr	Strongly positive, 4+.
	4	F	1 g	+	0	0	+	+	±	Strongly positive, 5+.
	5	M	1 g	+	0	0	+	+	±	Strongly positive, 5+.
	1-5	M and F	1 g	+	0	0	+	+	±	Strongly positive, 5+.
	6	F	1 g	+	tr	0	+	+	±	Strongly positive, 4+.
	7	M	1 g	+	tr	0	+	+	±	Strongly positive, 4+.
	8	F	1 g	+	tr	0	+	+	±	Strongly positive, 4+.
	9	M	1 g	+	±	0	+	+	±	Strongly positive, 3+.
2	10	F	1 g	+	tr	0	+	+	±	Strongly positive, 4+.
	6-10	M and F	1 g	+	tr	0	+	+	±	Strongly positive, 4+.
	1	M	1 g	+	±	0	+	+	±	Strongly positive, 3+.
	2	F	1 g	+	±	0	+	+	±	Strongly positive, 3+.
	3	M	1 g	+	±	0	+	+	0	Weakly positive, 1+.
	4	F	1 g	+	+	0	+	+	±	Moderately positive, 2+.
	5	M	1 g	+	+	0	+	+	±	Moderately positive, 2+.
	1-5	M and F	1 g	+	+	0	+	+	±	Moderately positive, 2+.
	6	F	1 g	+	+	0	+	+	±	Moderately positive, 2+.
	7	M	1 g	+	+	0	+	+	±	Moderately positive, 2+.
3	8	F	1 g	+	+	0	+	+	±	Moderately positive, 2+.
	9	M	1 g	+	±	0	+	+	±	Strongly positive, 3+.
	10	F	1 g	+	±	0	+	+	±	Strongly positive, 3+.
	6-10	M and F	1 g	+	+	0	+	+	±	Moderately positive, 2+.

* Explanation: In all tables + = complete hemolysis; ± = hemolysis between 50% and 100%; tr = hemolysis less than 50%; 0 = no hemolysis.

TABLE 1—Continued
COMPARISON OF TEN GUINEA-PIG COMPLEMENTS

Number of Serum	Number of Complement	Sex of Guinea-Pig	Amboceptor per Tube Unit	Readings*						Results
				Antigen Tubes			Control Tubes			
				1	2	3	1'	2'	3'	
3	1	M	1 g	+	0	0	+	+	±	Strongly positive, 5+.
	2	F	1 g	+	tr	0	+	+	+	Strongly positive, 5+.
	3	M	1 g	+	tr	0	+	+	0	Moderately positive, 2+
	4	F	1 g	+	±	0	+	+	±	Strongly positive, 3+
	5	M	1 g	+	tr	0	+	+	±	Strongly positive, 4+.
	1-5	M and F	1 g	+	tr	0	+	+	±	Strongly positive, 4+.
	6	F	1 g	+	±	0	+	+	±	Strongly positive, 3+.
	7	M	1 g	+	±	0	+	+	±	Strongly positive, 3+.
	8	F	1 g	+	±	0	+	+	±	Strongly positive, 3+.
	9	M	1 g	+	±	0	+	+	+	Strongly positive, 3+.
4	10	F	1 g	+	tr	0	+	+	±	Strongly positive, 4+.
	6-10	M and F	1 g	+	±	0	+	+	±	Strongly positive, 3+.
	1	M	1 g	+	tr	0	+	+	±	Strongly positive, 4+.
	2	F	1 g	+	+	0	+	+	+	Strongly positive, 3+.
	3	M	1 g	+	tr	0	+	+	tr	Strongly positive, 3+.
	4	F	1 g	+	tr	0	+	+	±	Strongly positive, 4+.
	5	M	1 g	+	tr	0	+	+	±	Strongly positive, 4+.
	1-5	M and F	1 g	+	tr	0	+	+	±	Strongly positive, 4+.
	6	F	1 g	+	0	0	+	+	±	Strongly positive, 5+.
	7	M	1 g	+	0	0	+	+	±	Strongly positive, 5+.
5	8	F	1 g	+	0	0	+	+	±	Strongly positive, 5+.
	9	M	1 g	+	0	0	+	+	±	Strongly positive, 5+.
	10	F	1 g	+	0	0	+	+	±	Strongly positive, 5+.
	6-10	M and F	1 g	+	0	0	+	+	±	Strongly positive, 5+.
	1	M	1 g	+	±	0	+	+	±	Strongly positive, 3+.
	2	F	1 g	+	+	tr	+	+	+	Moderately positive, 2+.
	3	M	1 g	+	tr	0	+	+	tr	Strongly positive, 3+.
	4	F	1 g	+	tr	0	+	+	±	Strongly positive, 4+.
	5	M	1 g	+	±	0	+	+	±	Strongly positive, 3+.
	1-5	M and F	1 g	+	±	0	+	+	±	Strongly positive, 3+.
6	6	F	1 g	+	tr	0	+	+	±	Strongly positive, 4+.
	7	M	1 g	+	tr	0	+	+	±	Strongly positive, 4+.
	8	F	1 g	+	tr	0	+	+	±	Strongly positive, 4+.
	9	M	1 g	+	tr	0	+	+	±	Strongly positive, 4+.
	10	F	1 g	+	0	0	+	+	±	Strongly positive, 5+.
	6-10	M and F	1 g	+	tr	0	+	+	±	Strongly positive, 4+.
	1	M	1 g	+	±	0	+	+	±	Strongly positive, 3+.
	2	F	1 g	+	+	0	+	+	+	Strongly positive, 3+.
	3	M	1 g	+	tr	0	+	+	tr	Strongly positive, 3+.
	4	F	1 g	+	tr	0	+	+	±	Strongly positive, 4+.
7	5	M	1 g	+	tr	0	+	+	±	Strongly positive, 4+.
	1-5	M and F	1 g	+	tr	0	+	+	±	Strongly positive, 4+.
	6	F	1 g	+	tr	0	+	+	±	Strongly positive, 4+.
	7	M	1 g	+	tr	0	+	+	±	Strongly positive, 4+.
	8	F	1 g	+	0	0	+	+	±	Strongly positive, 5+.
	9	M	1 g	+	0	0	+	+	±	Strongly positive, 5+.
	10	F	1 g	±	0	0	+	+	±	Strongly positive, 6+.
	6-10	M and F	1 g	+	0	0	+	+	±	Strongly positive, 5+.
	1	M	1 g	±	0	0	+	+	±	Strongly positive, 6+.
	2	F	1 g	+	tr	0	+	+	+	Strongly positive, 5+.
8	3	M	1 g	+	0	0	+	+	0	Strongly positive, 3+.
	4	F	1 g	±	0	0	+	+	±	Strongly positive, 6+.
	5	M	1 g	±	0	0	+	+	±	Strongly positive, 6+.
	1-5	M and F	1 g	±	0	0	+	+	±	Strongly positive, 6+.
	6	F	1 g	+	0	0	+	+	±	Strongly positive, 5+.
	7	M	1 g	+	0	0	+	+	±	Strongly positive, 5+.
	8	F	1 g	+	tr	0	+	+	±	Strongly positive, 4+.
	9	M	1 g	+	tr	0	+	+	±	Strongly positive, 4+.
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	6-10	M and F	1 g	+	tr	0	+	+	±	Strongly positive, 4+.

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	2	F	1 g	+	±	0	+	+	+	Strongly positive,	4+.
	3	M	1 g	+	tr	0	+	+	tr	Strongly positive,	3+.
	4	F	1 g	+	tr	0	+	+	±	Strongly positive,	4+.
	5	M	1 g	+	tr	0	+	+	±	Strongly positive,	4+.
	1-5	M and F	1 g	+	tr	0	+	+	±	Strongly positive,	4+.
	6	F	1 g	+	tr	0	+	+	±	Strongly positive,	4+.
	7	M	1 g	+	±	0	+	+	±	Strongly positive,	3+.
	8	F	1 g	+	+	0	+	+	±	Moderately positive,	2+.
	9	M	1 g	+	±	0	+	+	±	Strongly positive,	3+.
9	10	F	1 g	+	tr	0	+	+	±	Strongly positive,	4+.
	6-10	M and F	1 g	+	±	0	+	+	±	Strongly positive,	3+.
	1	M	1 g	+	0	0	+	+	±	Strongly positive,	5+.
	2	F	1 g	+	tr	0	+	+	+	Strongly positive,	5+.
	3	M	1 g	+	tr	0	+	+	tr	Strongly positive,	3+.
	4	F	1 g	+	0	0	+	+	±	Strongly positive,	5+.
	5	M	1 g	±	0	0	+	+	±	Strongly positive,	6+.
	1-5	M and F	1 g	±	0	0	+	+	±	Strongly positive,	6+.
	6	F	1 g	+	tr	0	+	+	±	Strongly positive,	4+.
	7	M	1 g	+	tr	0	+	+	±	Strongly positive,	4+.
10	8	F	1 g	+	±	0	+	+	±	Strongly positive,	3+.
	9	M	1 g	+	tr	0	+	+	±	Strongly positive,	4+.
	10	F	1 g	+	0	0	+	+	±	Strongly positive,	5+.
	6-10	M and F	1 g	+	0	0	+	+	±	Strongly positive,	5+.
	1	M	1 g	+	tr	0	+	+	±	Strongly positive,	4+.
	2	F	1 g	+	tr	0	+	+	+	Strongly positive,	5+.
	3	M	1 g	+	0	0	+	+	tr	Strongly positive,	4+.
	4	F	1 g	tr	0	0	+	+	tr	Strongly positive,	6+.
	5	M	1 g	±	0	0	+	+	±	Strongly positive,	6+.
	1-5	M and F	1 g	±	0	0	+	+	±	Strongly positive,	6+.
11	6	F	1 g	±	0	0	+	+	±	Strongly positive,	6+.
	7	M	1 g	+	0	0	+	+	±	Strongly positive,	5+.
	8	F	1 g	+	0	0	+	+	tr	Strongly positive,	4+.
	9	M	1 g	+	0	0	+	+	±	Strongly positive,	5+.
	10	F	1 g	±	0	0	+	+	±	Strongly positive,	6+.
	6-10	M and F	1 g	±	0	0	+	+	±	Strongly positive,	6+.
	1	M	1 g	+	0	0	+	+	±	Strongly positive,	5+.
	2	F	1 g	+	0	0	+	+	+	Strongly positive,	6+.
	3	M	1 g	±	0	0	+	+	±	Strongly positive,	5+.
	4	F	1 g	0	0	0	+	+	tr	Strongly positive,	8+.
12	5	M	1 g	tr	0	0	+	+	±	Strongly positive,	8+.
	1-5	M and F	1 g	±	0	0	+	+	±	Strongly positive,	6+.
	6	F	1 g	tr	0	0	+	+	±	Strongly positive,	8+.
	7	M	1 g	±	0	0	+	+	±	Strongly positive,	8+.
	8	F	1 g	±	0	0	+	+	±	Strongly positive,	6+.
	9	M	1 g	±	0	0	+	+	±	Strongly positive,	6+.
	10	F	1 g	tr	0	0	+	+	±	Strongly positive,	8+.
	6-10	M and F	1 g	tr	0	0	+	+	±	Strongly positive,	8+.
	1	M	1 g	+	tr	0	+	+	±	Strongly positive,	4+.
	2	F	1 g	+	tr	0	+	+	+	Strongly positive,	5+.
12	3	M	1 g	+	0	0	+	+	0	Strongly positive,	3+.
	4	F	1 g	±	0	0	+	+	tr	Strongly positive,	5+.
	5	M	1 g	±	0	0	+	+	±	Strongly positive,	5+.
	1-5	M and F	1 g	+	0	0	+	+	±	Strongly positive,	5+.
	6	F	1 g	+	0	0	+	+	±	Strongly positive,	5+.
	7	M	1 g	+	tr	0	+	+	±	Strongly positive,	4+.
	8	F	1 g	+	tr	0	+	+	±	Strongly positive,	4+.
	9	M	1 g	+	±	0	+	+	±	Strongly positive,	3+.
	10	F	1 g	+	tr	0	+	+	±	Strongly positive,	4+.
	6-10	M and F	1 g	+	0	0	+	+	±	Strongly positive,	5+.

Table 1 shows the reactivating power and the fixability of the 10 complements. The results obtained varied greatly. Complement 3 showed poor fixability and poor reactivating power. Serum 2 gave 3+ with Complements 1 and 2 and 1+ with Complement 3. Poor fixability was not uniform with all the serums because Serum 5 fixed Complement 3 better than it did Complement 2. The mixture of 5 complements gave a fair average of the results obtained with the complements singly. There was no difference between complement from male and from female guinea-pigs.

TEST 2

Five human complements (Complements 1-5, inclusive) were tested on Human Serums 7-12, inclusive. These were the same serums that were used in Test 1. The complements were used in doses of 0.2, 0.1 and 0.05 cc per test tube. Noguchi⁹ recommends 0.1 cc of human complement per test tube.

TABLE 2
COMPARISON OF HUMAN COMPLEMENTS

Number of Serum	Number of Complement	Sex of Guinea-Pig	Amboceptor per Tube Unit	Readings*						Results	
				Antigen Tubes			Control Tubes				
				1	2	3	1'	2'	3'		
7	1	M	1 g	+	+	0	+	+	tr	Weakly positive,	1+.
	2	F	1 g	+	+	tr	+	+	±	Weakly positive,	1+.
	3	M	1 g	+	+	tr	+	+	±	Weakly positive,	1+.
	4	F	1 g	+	+	tr	+	+	±	Weakly positive,	1+.
	5	M	1 g	+	+	tr	+	+	±	Weakly positive,	1+.
	1-5	M and F	1 g	+	+	tr	+	+	±	Weakly positive,	1+.
8	1	M	1 g	+	+	0	+	+	tr	Weakly positive,	1+.
	2	F	1 g	+	+	tr	+	+	±	Weakly positive,	1+.
	3	M	1 g	+	+	tr	+	+	±	Weakly positive,	1+.
	4	F	1 g	+	+	tr	+	+	±	Weakly positive,	1+.
	5	M	1 g	+	+	tr	+	+	±	Weakly positive,	1+.
	1-5	M and F	1 g	+	+	tr	+	+	±	Weakly positive,	1+.
9	1	M	1 g	+	+	0	+	+	tr	Weakly positive,	1+.
	2	F	1 g	+	+	tr	+	+	±	Weakly positive,	1+.
	3	M	1 g	+	+	tr	+	+	±	Weakly positive,	1+.
	4	F	1 g	+	+	tr	+	+	±	Weakly positive,	1+.
	5	M	1 g	+	+	tr	+	+	±	Weakly positive,	1+.
	1-5	M and F	1 g	+	+	tr	+	+	±	Weakly positive,	1+.
10	1	M	1 g	+	±	0	+	+	tr	Moderately positive,	2+.
	2	F	1 g	+	±	0	+	+	tr	Moderately positive,	2+.
	3	M	1 g	+	+	0	+	+	±	Moderately positive,	2+.
	4	F	1 g	+	+	0	+	+	±	Moderately positive,	2+.
	5	M	1 g	+	+	0	+	+	±	Moderately positive,	2+.
	1-5	M and F	1 g	+	+	0	+	+	±	Moderately positive,	2+.
11	1	M	1 g	+	±	0	+	+	tr	Moderately positive,	2+.
	2	F	1 g	+	±	0	+	+	tr	Moderately positive,	2+.
	3	M	1 g	+	+	0	+	+	±	Moderately positive,	2+.
	4	F	1 g	+	±	0	+	+	±	Strongly positive,	3+.
	5	M	1 g	+	+	0	+	+	±	Moderately positive,	2+.
	1-5	M and F	1 g	+	+	0	+	+	±	Moderately positive,	2+.
12	1	M	1 g	+	±	0	+	+	tr	Moderately positive,	2+.
	2	F	1 g	+	+	0	+	+	tr	Weakly positive,	1+.
	3	M	1 g	+	+	0	+	+	±	Moderately positive,	2+.
	4	F	1 g	+	+	0	+	+	±	Moderately positive,	2+.
	5	M	1 g	+	+	0	+	+	±	Moderately positive,	2+.
	1-5	M and F	1 g	+	+	0	+	+	±	Moderately positive,	2+.

The results obtained with the human complements are shown in Table 2. These were more uniform than those obtained with guinea-pig complement but were much weaker. With a mixture of 5 guinea-pig complements Serum 7 gave 6+ and with a mixture of 5 human complements it gave 1+. Serum 8 gave 4+ and 1+; Serum 9 gave 6+ and 1+; Serum 10 gave 6+ and 2+; Serum 11 gave 6+ and 2+, and Serum 12 gave 5+ and 2+.

TABLE 3
THE TEST AS RECOMMENDED BY NOGUCHI

Number of Serum	Number of Complement	Sex of Person	Amboceptor per Tube Unit	Readings*						Results	
				Antigen Tubes			Control Tubes				
				1	2	3	1'	2'	3'		
7	1·5	M and F	1 g	+	+	±	+	+	±	Negative,	—.
8	1·5	M and F	1 g	+	+	±	+	+	±	Negative,	—.
9	1·5	M and F	1 g	+	+	±	+	+	±	Negative,	—.
10	1·5	M and F	1 g	+	+	±	+	+	±	Negative,	—.
11	1·5	M and F	1 g	+	+	tr	+	+	±	Weakly positive,	1+.
12	1·5	M and F	1 g	+	+	±	+	+	±	Negative,	—.

TEST 3

Noguchi⁹ recently advocated the use of human complement instead of guinea-pig complement with heated human serum. The first incubation he conducts in the water-bath at 37 C. for 30 minutes. In order to show how that method compares with our regular method Serums 7-12, inclusive, were tested. These serums came from patients that were known to be syphilitic.

TABLE 4
THE EFFECT OF NORMAL HUMAN SERUM ON THE SO-CALLED SYPHILITIC ANTIBODY

Number of Serum	Diluent	Comple-ment	Ambo-ceptor per Tube Unit	Readings*						Results
				Antigen Tubes			Control Tubes			
				1	2	3	1'	2'	3'	
7	S. S.	G-P.	1 g	+	+	0	+	+	±	Moderately positive, 2+.
	F. H. S.	G-P.	1 g	+	+	±	+	+	±	Negative, —.
	H. H. S.	G-P.	1 g	+	+	±	+	+	±	Negative, —.
8	S. S.	G-P.	1 g	+	+	tr	+	+	±	Weakly positive, 1+.
	F. H. S.	G-P.	1 g	+	+	±	+	+	±	Negative, —.
	H. H. S.	G-P.	1 g	+	+	±	+	+	±	Negative, —.
9	S. S.	G-P.	1 g	+	+	0	+	+	±	Moderately positive, 2+.
	F. H. S.	G-P.	1 g	+	+	±	+	+	±	Negative, —.
	H. H. S.	G-P.	1 g	+	+	±	+	+	±	Negative, —.
10	S. S.	G-P.	1 g	+	±	0	+	+	±	Strongly positive, 3+.
	F. H. S.	G-P.	1 g	+	+	±	+	+	±	Negative, —.
	H. H. S.	G-P.	1 g	+	+	±	+	+	±	Negative, —.
11	S. S.	G-P.	1 g	+	tr	0	+	+	tr	Strongly positive, 3+.
	F. H. S.	G-P.	1 g	+	+	tr	+	+	tr	Negative, —.
	H. H. S.	G-P.	1 g	+	+	tr	+	+	tr	Negative, —.
12	S. S.	G-P.	1 g	+	+	0	+	+	±	Moderately positive, 2+.
	F. H. S.	G-P.	1 g	+	+	±	+	+	±	Negative, —.
	H. H. S.	G-P.	1 g	+	+	±	+	+	±	Negative, —.

Table 3 shows the results obtained with the method recently advocated by Noguchi. Serums 7, 8, 9, 10 and 12 gave negative results and Serum 11 gave a weakly positive result, 1+. As Table 1 shows, our regular method gave positive results. Serums 7, 8, 9, 10, 11 and 12 gave 6+, 4+, 6+, 6+, 6+ and 5+, respectively.

TEST 4

Having found that human complement gives much weaker positive results than does guinea-pig complement the effect of normal human serum on syphilitic human serum was studied on 6 syphilitic serums. Three portions were taken from each of Serums 7-12, inclusive; one portion of each serum was diluted with an equal volume of salt solution containing 50% glycerol (S. S.), one portion was diluted with an equal volume of glycerolated fresh human serum (F. H. S.) and the other portion was mixed with an equal volume of glycerolated heated human serum (H. H. S.). At about 24 hours after these serums had been mixed these mixtures were tested by our regular method.

Table 4 shows the results obtained with Serums 7, 8, 9, 10, 11 and 12 after they had been diluted with an equal volume of salt solution and with an equal volume of normal human serum. Diluted with an equal volume of salt solution these serums gave 2+, 1+, 2+, 3+, 3+ and 2+, respectively, while all the portions diluted with an equal volume of human serum fresh or heated gave negative results.

TABLE 5

THE EFFECT OF GUINEA-PIG SERUM ON THE SO-CALLED SYPHILITIC ANTIBODY

Number of Serum	Diluent	Comple-ment	Ambo-ceptor per Tube Unit	Readings*						Results
				Antigen Tubes			Control Tubes			
				1	2	3	1'	2'	3'	
8	S. S.	G-P	1 g	+	±	0	+	+	tr	Moderately positive, 2+.
	G-P. S. 1	G-P	1 g	+	+	±	+	+	±	Negative, —.
	G-P. S. 2	G-P	1 g	+	+	±	+	+	±	Negative, —.
	G-P. S. 3	G-P	1 g	+	+	±	+	+	±	Negative, —.
9	S. S.	G-P	1 g	+	tr	0	+	+	tr	Strongly positive, 3+.
	G-P. S. 1	G-P	1 g	+	+	±	+	+	±	Negative, —.
	G-P. S. 2	G-P	1 g	+	+	±	+	+	±	Negative, —.
	G-P. S. 3	G-P	1 g	+	+	±?	+	+	±	Faintly positive, ±.
10	S. S.	G-P	1 g	+	tr	0	+	+	tr	Strongly positive, 3+.
	G-P. S. 1	G-P	1 g	+	+	±	+	+	±	Negative, —.
	G-P. S. 2	G-P	1 g	+	+	±	+	+	±	Negative, —.
	G-P. S. 3	G-P	1 g	+	+	±?	+	+	±	Faintly positive, ±.
11	S. S.	G-P	1 g	+	tr	0	+	+	tr	Strongly positive, 3+.
	G-P. S. 1	G-P	1 g	+	+	±	+	+	±	Negative, —.
	G-P. S. 2	G-P	1 g	+	+	±?	+	+	±	Faintly positive, ±.
	G-P. S. 3	G-P	1 g	+	+	±?	+	+	±	Faintly positive, ±.
12	S. S.	G-P	1 g	+	tr	0	+	+	tr	Strongly positive, 3+.
	G-P. S. 1	G-P	1 g	+	+	±	+	+	±	Negative, —.
	G-P. S. 2	G-P	1 g	+	+	±	+	+	±	Negative, —.
	G-P. S. 3	G-P	1 g	+	+	±	+	+	±	Negative, —.

TEST 5

The work of Test 4 was repeated with heated guinea-pig serum (G-P. S.) instead of normal human serum as diluent.

The effect of guinea-pig serum on syphilitic human serum is shown in Table 5. Mixed with an equal volume of salt solution Serums 8, 9, 10, 11

and 12 gave 2+, 3+, 3+, 3+ and 3+, respectively, while the same serums mixed with equal volumes of guinea-pig serums gave negative or nearly negative results.

TEST 6

The rate at which the complement-binding power disappears from syphilitic human serum when mixed with normal human or guinea-pig serum was observed on Serums 10 and 11. Three portions were taken of each serum; one portion was diluted with an equal volume of salt solution (S. S.) containing 50% glycerol; one portion was diluted with an equal volume of glycerolated human serum (H. S.), and the other portion was mixed with an equal volume of glycerolated guinea-pig serum. Each portion was tested at the end of 2 hours, 4 hours and 6 hours.

TABLE 6
THE RATE OF DISAPPEARANCE OF COMPLEMENT-BINDING POWER

Number of Serum	Diluent	Time	Ambo- ceptor per Tube Unit	Readings*						Results
				Antigen Tubes			Control Tubes			
				1	2	3	1'	2'	3'	
10	S. S.	2	1 g	+	+	0	+	+	±	Moderately positive, 2+.
	H. S.	2	1 g	+	+	tr	+	+	±	Weakly positive, 1+.
	G.-P. S.	2	1 g	+	+	±?	+	+	±	Faintly positive, ±.
	S. S.	4	1 g	+	+	0	+	+	±	Moderately positive, 2+.
	H. S.	4	1 g	+	+	±?	+	+	±	Faintly positive, ±.
	G.-P. S.	4	1 g	+	+	±	+	+	±	Negative, —.
	S. S.	6	1 g	+	+	0	+	+	±	Moderately positive, 2+.
	H. S.	6	1 g	+	+	±?	+	+	±	Faintly positive, ±.
G.-P. S.	6	1 g	+	+	±	+	+	±	Negative, —.	
11	S. S.	2	1 g	+	±	0	+	+	±	Strongly positive, 3+.
	H. S.	2	1 g	+	+	tr	+	+	±	Weakly positive, 1+.
	G.-P. S.	2	1 g	+	+	tr	+	+	±	Weakly positive, 1+.
	S. S.	4	1 g	+	±	0	+	+	±	Strongly positive, 3+.
	H. S.	4	1 g	+	+	tr	+	+	±	Weakly positive, 1+.
	G.-P. S.	4	1 g	+	+	±?	+	+	±	Faintly positive, ±.
	S. S.	6	1 g	+	±	0	+	+	±	Strongly positive, 3+.
	H. S.	6	1 g	+	+	±?	+	+	±	Faintly positive, ±.
	G.-P. S.	6	1 g	+	+	±?	+	+	±	Faintly positive, ±.

Table 6 shows the rate at which the complement-binding power disappeared from Serums 10 and 11. At the end of 2 hours about 50% of the complement-binding power had disappeared from the portion mixed with normal human serum and about 75% from the portion mixed with guinea-pig serum. At the end of 4 hours the normal human serum had neutralized about 75% of Serum 10 and the guinea-pig serum had completely neutralized it. With Serum 11 neutralization was a trifle slower.

TEST 7

Deterioration of guinea-pig complement was determined in the refrigerator ranging from 10-15 C. Three guinea-pig serums (Serums 11, 12 and 13) were each divided into 2 portions, A and B. Portion A was left on the clot and Portion B was removed from the clot and was kept in a sterile test tube. Each serum was tested singly and the three serums were mixed and the mixture was also tested. The mixture was designated X; X A was mixed

immediately before the test while X B was mixed a few hours after the bleeding; the mixture was kept in the refrigerator and was tested from day to day. In these tests the quantity of amboceptor was kept constant.

TABLE 7
DETERIORATION OF COMPLEMENT IN THE REFRIGERATOR

Number of Comple- ment	Portions A=On Clot B=Off Clot	Age Days	Number of Serum	Readings*						Results
				Antigen Tubes			Control Tubes			
				1	2	3	1'	2'	3'	
11	A	1	10	±	0	0	+	+	tr	Strongly positive, 5+.
	B	1	10	±	0	0	+	+	tr	Strongly positive, 5+.
	A	2	10	±	0	0	+	+	tr	Strongly positive, 5+.
	B	2	10	+	0	0	+	+	tr	Strongly positive, 4+.
	A	3	10	±	0	0	+	+	0	Strongly positive, 4+.
	B	3	10	±	0	0	+	±	0	Strongly positive, 3+.
	A	4	10	±	0	0	+	+	0	Strongly positive, 4+.
	B	4	10	±	0	0	+	tr	0	Moderately positive, 2+.
	A	5	10	±	0	0	+	±	0	Strongly positive, 3+.
B	5	10	tr	0	0	±	tr	0	Positive (Unfit).	
12	A	1	10	±	0	0	+	+	±	Strongly positive, 6+.
	B	1	10	±	0	0	+	+	±	Strongly positive, 6+.
	A	2	10	±	0	0	+	+	tr	Strongly positive, 5+.
	B	2	10	+	0	0	+	+	tr	Strongly positive, 4+.
	A	3	10	±	0	0	+	+	tr	Strongly positive, 5+.
	B	3	10	±	0	0	+	+	0	Strongly positive, 4+.
	A	4	10	±	0	0	+	+	tr	Strongly positive, 5+.
	B	4	10	±	0	0	+	±	0	Strongly positive, 3+.
	A	5	10	±	0	0	+	+	tr	Strongly positive, 5+.
B	5	10	±	0	0	±	tr	0	Unfit	
13	A	1	10	±	0	0	+	+	±	Strongly positive, 6+.
	B	1	10	±	0	0	+	+	±	Strongly positive, 6+.
	A	2	10	±	0	0	+	+	tr	Strongly positive, 5+.
	B	2	10	+	0	0	+	+	tr	Strongly positive, 4+.
	A	3	10	±	0	0	+	+	0	Strongly positive, 4+.
	B	3	10	+	0	0	+	±	0	Moderately positive, 2+.
	A	4	10	+	0	0	+	+	0	Strongly positive, 3+.
	B	4	10	+	tr	0	±	tr	0	Unfit
	A	5	10	±	0	0	+	±	0	Strongly positive, 3+.
B	5	10	+	0	0	±	tr	0	Unfit	
X	A	1	10	±	0	0	+	+	±	Strongly positive, 6+.
	B	1	10	±	0	0	+	+	±	Strongly positive, 6+.
	A	2	10	±	0	0	+	+	tr	Strongly positive, 5+.
	B	2	10	±	0	0	+	+	tr	Strongly positive, 5+.
	A	3	10	±	0	0	+	+	0	Strongly positive, 4+.
	B	3	10	±	0	0	+	±	tr	Strongly positive, 3+.
	A	4	10	±	0	0	+	±	tr	Strongly positive, 3+.
	B	4	10	±	tr	0	±	tr	0	Negative (Unfit).
	A	5	10	±	0	0	+	±	0	Strongly positive, 3+.
B	5	10	±	0	0	±	tr	0	Unfit	

The rate at which these 3 complements deteriorated in the refrigerator is shown in Table 7. Portion A remained fairly constant for 3 days while Portion B of the serums singly and in mixture showed considerable deterioration on the 3rd day. After the 3rd day deterioration of Portion A also became noticeable. Deterioration took place in the fixability of the complement and in the power to reactivate the hemolytic amboceptor.

TEST 8

In accordance with the method described by Ramy,² 3 fresh guinea-pig serums were diluted with a 10% solution of sodium acetate. A few hours after the guinea-pigs had been bled each serum was divided into 2 portions,

A and B. Portion A was left on the clot and no preservative was added. Portion B was pipetted off the clot, was centrifuged and 4 parts of the clear serum were mixed with 6 parts of a 10% solution of sodium acetate in salt solution (9 gm. NaCl per liter). The serums were tested singly and in the form of a mixture of the 3 serums which is designated as X. X A was mixed immediately before testing and X B was mixed immediately after Portion B had been mixed with the sodium acetate solution. The serums were kept in the refrigerator and were tested at short intervals.

TABLE 8
COMPLEMENT PRESERVED WITH SODIUM ACETATE

Number of Complement	Portions A=Not Pre- served B=Pre- served	Age Days	Number of Serum	Readings*						Results	
				Antigen Tubes			Control Tubes				
				1	2	3	1'	2'	3'		
14	A	1	10	±	0	0	+	+	±	Strongly positive,	6+
	B	1	10	±	0	0	+	+	±	Strongly positive,	6+
	B	2	10	+	0	0	+	+	±	Strongly positive,	5+
	A	3	10	±	0	0	+	+	±	Strongly positive,	6+
	B	3	10	+	tr	0	+	+	±	Strongly positive,	4+
	B	4	10	+	0	0	+	+	tr	Strongly positive,	4+
	A	6	10	±	0	0	±	tr	0	Unfit.	
	B	6	10	+	tr	0	+	±	tr	Positive.	
	B	8	10	+	tr	0	+	±	tr	Positive.	
	B	11	10	+	tr	0	+	±	tr	Positive.	
15	B	16	10	+	tr	0	+	±	tr	Positive.	
	A	1	10	±	0	0	+	+	±	Strongly positive,	6+
	B	1	10	±	0	0	+	+	±	Strongly positive,	6+
	B	2	10	+	0	0	+	+	±	Strongly positive,	5+
	A	3	10	tr	0	0	+	+	tr	Strongly positive,	6+
	B	3	10	+	tr	0	+	+	±	Strongly positive,	4+
	B	4	10	+	0	0	+	+	tr	Strongly positive,	4+
	A	6	10	+	tr	0	+	±	tr	Positive.	
	B	6	10	+	±	0	+	+	±	Strongly positive,	3+
	B	8	10	+	tr	0	+	+	tr	Strongly positive,	3+
16	B	11	10	+	tr	0	+	+	tr	Strongly positive,	3+
	B	16	10	±	tr	0	±	±	tr	Positive.	
	A	1	10	±	0	0	+	+	±	Strongly positive,	6+
	B	1	10	±	0	0	+	+	±	Strongly positive,	6+
	B	2	10	+	0	0	+	+	±	Strongly positive,	5+
	A	3	10	±	0	0	+	+	±	Strongly positive,	6+
	B	3	10	+	tr	0	+	+	±	Strongly positive,	4+
	B	4	10	+	tr	0	+	+	±	Strongly positive,	4+
	A	6	10	+	±	tr	+	+	±	Positive.	
	B	6	10	+	±	0	+	+	±	Strongly positive,	3+
X	B	8	10	+	±	0	+	+	±	Strongly positive,	3+
	B	11	10	+	tr	0	+	+	tr	Strongly positive,	3+
	B	16	10	+	tr	0	+	±	tr	Positive.	
	A	1	10	±	0	0	+	+	±	Strongly positive,	6+
	B	1	10	±	0	0	+	+	±	Strongly positive,	6+
	B	2	10	+	0	0	+	+	±	Strongly positive,	5+
	A	3	10	±	0	0	+	+	±	Strongly positive,	6+
	B	3	10	+	tr	0	+	+	±	Strongly positive,	4+
	B	4	10	+	0	0	+	+	tr	Strongly positive,	4+
	A	6	10	+	tr	0	+	+	tr	Strongly positive,	3+
	B	6	10	+	±	0	+	+	±	Strongly positive,	3+
	B	8	10	+	tr	0	+	+	tr	Strongly positive,	3+
	B	11	10	+	tr	0	+	+	tr	Strongly positive,	3+
	B	16	10	+	tr	0	±	±	tr	Unfit.	

Table 8 shows the results obtained by comparing complement preserved by Ramy's method with complement that was without preservative. On the 1st day after the serums had been obtained Portions A and B gave results that were

identical. On the 3rd day Portion A of each complement and of the mixture X gave 6+ and Portion B gave 4+. On the 6th day Portion A of each serum was practically unfit while Portion B gave 3+. On the 8th day and on the 11th day all Portions B gave 3+ while on the 16th day all were unfit. In the serum mixed with sodium acetate solution complement deterioration began earlier than in the unpreserved serum but it progressed somewhat slower. While in plain serum, Portion A, the complement showed full strength on the 3rd day it had lost one-third of its fixability in the preserved serum, Portion B.

TABLE 9
GLYCEROLATED COMPLEMENT COMPARED WITH NONGLYCEROLATED COMPLEMENT

Number of Complement	Portions A=On Clot B=Off Clot C=Glycerolated	Age Days	Number of Serum	Readings*						Results
				Antigen Tubes			Control Tubes			
				1	2	3	1'	2'	3'	
17	A	1	10	+	0	0	+	+	±	Strongly positive, 5+.
	B	1	10	+	0	0	+	+	±	Strongly positive, 5+.
	C	1	10	±	0	0	+	+	±	Strongly positive, 6+.
	A	3	10	+	tr	0	+	+	±	Strongly positive, 4+.
	B	3	10	+	tr	0	+	+	±	Strongly positive, 4+.
	C	3	10	±	0	0	+	+	±	Strongly positive, 6+.
	A	7	10	+	tr	0	+	+	tr	Strongly positive, 3+.
	B	7	10	+	tr	0	+	tr	0	Negative.
	C	7	10	+	±	0	+	+	±	Strongly positive, 3+.
	C	10	10	±	0	0	±	±	tr	Unfit.
18	A	1	10	±	0	0	+	+	±	Strongly positive, 6+.
	B	1	10	±	0	0	+	+	±	Strongly positive, 6+.
	C	1	10	±	0	0	+	+	±	Strongly positive, 6+.
	A	3	10	+	0	0	+	+	±	Strongly positive, 5+.
	B	3	10	+	tr	0	+	+	±	Strongly positive, 4+.
	C	3	10	tr	0	0	+	+	±	Strongly positive, 8+.
	A	7	10	+	tr	0	+	+	tr	Strongly positive, 3+.
	B	7	10	+	tr	0	+	tr	0	Negative.
	C	7	10	+	0	0	+	+	±	Strongly positive, 5+.
	C	10	10	tr	0	0	±	±	tr	Unfit.
19	A	1	10	±	0	0	+	+	±	Strongly positive, 6+.
	B	1	10	±	0	0	+	+	±	Strongly positive, 6+.
	C	1	10	±	0	0	+	+	±	Strongly positive, 6+.
	A	3	10	+	0	0	+	+	±	Strongly positive, 5+.
	B	3	10	+	tr	0	+	+	±	Strongly positive, 4+.
	C	3	10	tr	0	0	+	+	±	Strongly positive, 8+.
	A	7	10	+	tr	0	+	+	tr	Strongly positive, 3+.
	B	7	10	+	tr	0	+	tr	0	Negative.
	C	7	10	+	0	0	+	+	±	Strongly positive, 5+.
	C	10	10	tr	0	0	±	±	tr	Unfit.
X	A	1	10	±	0	0	+	+	±	Strongly positive, 6+.
	B	1	10	±	0	0	+	+	±	Strongly positive, 6+.
	C	1	10	±	0	0	+	+	±	Strongly positive, 6+.
	A	3	10	+	0	0	+	+	±	Strongly positive, 5+.
	B	3	10	+	tr	0	+	+	±	Strongly positive, 4+.
	C	3	10	± ^y	0	0	+	+	±	Strongly positive, 7+.
	A	7	10	+	tr	0	+	+	tr	Strongly positive, 3+.
	B	7	10	+	tr	0	+	tr	0	Negative.
	C	7	10	+	0	0	+	+	±	Strongly positive, 5+.
	C	10	10	tr	0	0	±	±	tr	Unfit.

TEST 9

Glycerol as a preservative for complement was studied on 3 guinea-pig serums, Complements 17, 18 and 19. A few hours after bleeding each serum was divided into 3 portions, A, B and C. Portion A was left on the clot, Portion B was pipetted off the clot and was put into a sterile test tube.

Portion C was pipetted off the clot, was put into a sterile tube and 1 part of sterilized glycerol was mixed with 3 parts of serum. All serums were tested singly and in a mixture composed of the 3 serums. The mixture is designated as X. X A was mixed immediately before testing, while X B and X C were prepared a few hours after the guinea-pigs had been bled. All complement serums were kept in the refrigerator until used, and were tested on the 1st, 3rd and 7th days after having been obtained from the guinea-pigs.

Table 9 shows the results obtained with glycerolated complement as compared with nonglycerolated complement. Portion A showed a slight loss of fixability on the 3rd day, Portion B had lost a trifle more than Portion A, and Portion C was bound better on the 3rd day than on the 1st day. On the 7th day Portion A was fixed or bound very poorly, Portion B was not bound at all, while Portion C gave results nearly equal to those obtained on the 1st day. Portions A and B were not tested after the 7th day while Portion C was found to be unfit on the 10th day.

SUMMARY AND CONCLUSIONS

The complements of fresh guinea-pig serum varied greatly in fixability. While one complement gave 6+ with a certain human serum another complement gave only 3+ with the same serum, other things having been equal. Another serum gave 3+ with one complement and 1+ with another complement. This variation in fixability was not due to complement alone when a complement showed poor fixability with a certain human serum the same complement was well fixed by other human serums. A mixture of 5 complement serums gave fairly uniform results. These results show clearly the unreliability of a single complement serum.

Human complement gave much weaker positive results than did guinea-pig complement. Noguchi's recent method of doing the Wassermann reaction gave negative results with serum that was known to be from syphilitic persons and gave 6+ by our regular method. Normal human serum and guinea-pig serum, respectively, were mixed with syphilitic human serum giving 6+ result with the Wassermann reaction; on testing them again 24 hours later negative results were obtained while controls which were diluted similarly with salt solution gave positive results. Neutralization of complement-fixing power by normal human serum or by guinea-pig serum progressed fairly rapidly, 50% was neutralized in 2 hours. At the end of 4 hours neutralization of complement-fixing power was sometimes complete, especially by guinea-pig serum, which was a trifle more effective than the human serum. A neutralization test of this kind may become of value in

detecting syphilitic serum which no longer gives a positive result by the Wassermann reaction alone. Future observations must determine its value.

Guinea-pig complement left on the clot and kept in the refrigerator remained fairly constant for 3 days, while complements which had been removed from the clots deteriorated more rapidly. Deterioration took place in two directions, the complement lost fixability and it lost the power to reactivate the hemolytic amboceptor. Complements with which 6+ was obtained about 24 hours after bleeding gave negative results with the same human serums 6 days later. Fixability disappeared more rapidly than did the power to reactivate hemolytic amboceptor.

Ramy's method of preserving complement by sodium acetate was a total failure in my hands. Fresh guinea-pig serum mixed in proportion of 4:6 with a 10% solution of sodium acetate in salt solution began to deteriorate immediately. In 3 days it lost about 33% fixability while the control portion to which no preservative had been added remained constant. Although deterioration of preserved complement began earlier than of the control it progressed somewhat slower.

Glycerol seemed to prevent deterioration of complement for a few days. In complement preserved by glycerol fixability increased during the first 3 days and on the 7th day it still was nearly equal to the fresh serum.

FURTHER STUDIES OF PLEOMORPHIC STREPTOCOCCI- BIOLOGIC REACTIONS

WITH ONE PLATE

NOBLE P. SHERWOOD AND CORNELIA M. DOWNS

From the Department of Bacteriology, University of Kansas, Lawrence.

In a previous paper, one of us¹ described several pleomorphic strains of *Streptococcus salivarius* in tonsillitis, and in the course of routine work on meningococcus carriers, we made note of the relative frequency of pleomorphic streptococci and determined their classification. The organisms formed chains uniformly, under anaerobic conditions they remained small, and pleomorphism was constant under aerobic conditions. They had the same morphologic and cultural characteristics as many of the streptococci recently described by Rosenow and his associates² in poliomyelitis. In view of their interesting results we thought it worth while to compare the results of animal inoculations and immunological reactions. This seemed desirable as our results suggest an extensive distribution of the organisms. The cultural characteristics of the strains we studied are given in Table 1.

Probably the most satisfactory classification of streptococci that has been worked out so far is the one suggested by Holman³ (Table 2). It is quite evident that the organisms we are interested in would be classified according to Holman as *Streptococcus salivarius*. They would also be classified the same according to Andrews and Horder's⁴ classification, except that our strains are more or less pathogenic, whereas Andrews and Horder state that *Streptococcus salivarius* is nonpathogenic. *Streptococcus anginosus* is culturally the same as the *salivarius* except that it is hemolytic for several kinds of red blood cells.

We find that according to Holman's classification, Rosenow and his associates² have described 19 strains of *S. salivarius*, 21 strains of *S. mitis*, 6 strains of *S. fecalis* and several others that might or might not fit into Holman's classification, depending on capsule formation,

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¹ Sherwood, N. P.: *Kans. Univ. Science Bull.*, 1917, 10, p. 247.

² *Jour. Infect. Dis.*, 1918, 22, p. 313.

³ *Jour. Med. Research*, 1916, 34, p. 377.

⁴ *Lancet*, 1916, 2, p. 708.

TABLE 1
NONHEMOLYTIC PLEOMORPHIC STREPTOCOCCI

Colony Type on Dextrose or Plain Blood-Agar Plates	Gram Stain	Gela- tin Lique- fac- tion	Litmus Milk		Fermentation Reaction Acid								Solu- bility in Bile	Cap- sule
			Acid	Acid Coag- ula- tion	Dex- trose	Lac- tose	Man- nite	Sal- icin	Raffi- nose	Saccha- rose	Inu- lin			
Small discrete colonies, brown by both reflected and transmitted light with a zone of brownish discoloration around each colony.	+	0	+	Variable	+	+	—	—	+	+	—	0	0	

TABLE 2
STREPTOCOCCI-GRAM-POSITIVE COCCI IN CHAINS, NO CAPSULES

+Hemolysis-							
+Mannite-		+Lactose-		+Mannite-		+Lactose-	
+Salicin-	+Salicin-	+Salicin-	+Salicin-	+Salicin-	+Salicin-	+Salicin-	+Salicin-
Strep. infrequens	Strep. pyogenes	Strep. hemolyticus II	Strep. equi	Strep. fecalis	Strep. mitis	Strep. nonhemolyticus II	Strep. equinus
Strep. hemolyticus I	Strep. anginosus	Strep. hemolyticus III	Strep. subacidus	Strep. nonhemolyticus I	Strep. salivarius	Strep. nonhemolyticus III	Strep. ignavus

bile solubility and permanence of inulin fermentation. The decidedly pleomorphic strains as well as several at least of those used for animal inoculation, could be classified as *S. salivarius*. It has been our experience that practically all of the marked pleomorphic streptococci from the throat that show any degree of permanence of the tendency to produce involution forms, fall in this class.

In the course of a determination of meningococcus carriers we decided to note also the relative frequency of pleomorphic streptococci and to determine their classification and pathogenicity. We swabbed the nasopharynx of 130 schoolchildren and found pleomorphic streptococci present in 25%. During an epidemic of sore throat and pneumonia due to *S. hemolyticus* we found nonhemolytic pleomorphic streptococci in 19 of 78 throat cultures. Nasopharyngeal examinations for meningococci were made of 101 students and pleomorphic streptococci occurred in approximately 25%.

We used dextrose blood-agar plates. The medium was meat infusion agar with a reaction of 0.2% to phenolphthalein, and containing 2% dextrose and 5% whole blood. In view of the fact that dextrose interferes with hemolysis by some streptococci that are truly hemolytic, we carefully checked up this property on sugar-free blood agar. In determining the fermentations we found that sugar broths were not nearly so satisfactory as serum agar containing 2% of the desired carbohydrate and Andrade's indicator as used in the new Russell medium. In making up serum agar, we added 5 cc of serum, diluted 1:3 with distilled water and autoclaved, to each 100 cc of melted agar, and sterile carbohydrate solution in sufficient amount to make 2%, Andrade's indicator was added also. After thorough mixing the neck of the bottles was flamed and the medium poured directly into freshly sterilized test tubes, about 8 cc per tube. These were slanted as for Russell's medium and incubated to determine contamination. It was found advisable to make both surface and deep inoculation. Frequently acid production was noticed anaerobically but not aerobically. This is a satisfactory medium for pneumococci and also for quite delicately growing streptococci.

Twenty-four-hour cultures were used for animal inoculation. Rabbits weighing from 250-850 gm. mainly were used. The hair was clipped from the scalp and the animal put under light ether anesthesia. Working under sterile conditions the scalp was split longitudinally and drawn to one side. At first a small trephine was used, but later a hole was bored with a scalpel and the inoculation made through this opening. Inoculations of 0.25-0.5 cc of 24-hour cultures were made into the posterior part of the cerebrum in one set and in another set in the motor areas. Subdural inoculations were also made. In addition inoculations of 10-20 cc were made into the marginal ear vein. The weight and temperature were taken several times before the inoculations and at short intervals thereafter.

In all, 45 animals were inoculated; 29 with pleomorphic streptococci as follows: 8 intravenously, 18 intracerebrally in the posterior part of the cerebrum, 4 in the motor areas, and one subdurally. Six were inoculated intracerebrally with *B. dysenteriae*, *B. typhosus* and *B. coli*. In addition controls were inoculated intracerebrally and intravenously with sterile ascitic fluid dextrose broth and washings from sterile dextrose serum agar plates.

The results of our experiments are summarized in Table 3; the plus sign indicates positive and the minus sign negative results. It is realized that percentage figures in such a short series are not significant, but in view of the fact that others² in a similar series of animals used the percentage basis it seemed advisable for us to do so for purposes of comparison.

We see that the incubation period of the intravenously inoculated animals varied from 3-12 days, and that 50% showed a rise in temperature, tremor and loss of muscle tone. None, however, developed flaccid paralysis. Those inoculated intracerebrally had an incubation period of from 5-24 hours. Of these, 95.5% showed rise in temperature, 54.6% showed in addition loss of muscle tone, 4.55% developed flaccid paralysis, 22.75% showed rise in temperature only and 4.55% showed no change in temperature or other symptoms; 13.65%

TABLE 3

INJECTION OF RAUPTS

Organism Injected	Method of Inoculation	Dose in Temperature of 2-4 C	Symptoms				Prostration from Overwhelming Inoculation	Time before Symptoms Other Than Fever Develop	Remarks
			Rise in Temperature	Tremor	Loss of Muscle Tone	General Weakness	Flaccid Paralysis		
Pleomorphic streptococcus.....	Intrav.	10.0	+	+	+	..	—	3 days	Died in 15 days
Pleomorphic streptococcus.....	Intrav.	8.0	+	+	+	..	—	7 days	Recovered
Pleomorphic streptococcus.....	Intrav.	10.0	+	+	+	..	—	7 days	Recovered
Pleomorphic streptococcus.....	Intrav.	10.0	+	+	+	..	—	8 days	Died in 48 hours
Pleomorphic streptococcus.....	Intrav.	10.0	+	+	+	..	—	Died in 4 days
Pleomorphic streptococcus.....	Intrav.	10.0	—	—	—	..	—	Died
Pleomorphic streptococcus.....	Intrav.	10.0	+	+	+	..	—	12 days	Remained normal
Controls (4).....	Intrav.	10.0	—	—	—	..	—	Progressive recovery
Pleomorphic streptococcus.....	Intrac.	0.5	+	+	+	..	—	12 hours	Died in 48 hours
Pleomorphic streptococcus.....	Intrac.	0.25	+	+	+	..	—	24 hours	Recovered
Pleomorphic streptococcus.....	Intrac.	0.25	+	+	+	..	—	5 hours	Recovered
Pleomorphic streptococcus.....	Intrac.	0.5	+	+	+	..	—	24 hours	Recovered
Pleomorphic streptococcus.....	Intrac.	0.5	+	+	+	..	—	Died in 48 hours
Pleomorphic streptococcus.....	Intrac.	0.5	+	+	+	..	—	6 hours	Died during night
Pleomorphic streptococcus.....	Intrac.	0.5	+	+	+	..	—	Recovered
Pleomorphic streptococcus.....	Intrac.	0.25	+	+	+	..	—	Recovered
Pleomorphic streptococcus.....	Intrac.	0.25	+	+	+	..	—	5 hours	Recovered after 6 weeks
Pleomorphic streptococcus.....	Intrac.	0.25	+	+	+	..	—	5 hours	Remained normal
Pleomorphic streptococcus.....	Intrac.	0.25	+	+	+	..	—	Remained normal
Pleomorphic streptococcus.....	Intrac.	0.25	+	+	+	..	—	Died during night
Pleomorphic streptococcus.....	Intrac.	0.25	+	+	+	..	—	Died in convulsions
Pleomorphic streptococcus.....	Intrac.	0.5	+	+	+	..	—	after 24 hours
Pleomorphic streptococcus.....	Intrac.	0.5	+	+	+	..	—	Recovered
Pleomorphic streptococcus.....	Intrac.	0.5	+	+	+	..	—	12 hours	Recovered
Pleomorphic streptococcus.....	Intrac.	0.9	+	+	+	..	+	5 hours	Died after 30 hours
Pleomorphic streptococcus.....	Intrac.	0.25	+	+	+	..	—	5 hours	Recovered
Pleomorphic streptococcus.....	Intrac.	0.5	+	+	+	..	—	5 hours	Died after 48 hours
Pleomorphic streptococcus.....	Intrac.	0.5	+	+	+	..	—	18 hours	Not completely recovered in 3 months
Pleomorphic streptococcus.....	Intrac.	0.5	+	+	+	..	—	18 hours	Remained normal
Controls (4).....	Intrac.	0.5	—	—	—	..	—	Died in 18 hours, irritable marked toxic symptoms
B. dysenteriae.....	Intrac.	0.5	—	—	—	..	—	Marked irritability, retraction of head, spastic paralysis of fore legs. Died in 12 hours (chloroformed)
B. typhosus.....	Intrac.	0.5	—	—	—	..	—	Toxic symptoms, died in 15 hours
B. coli.....	Intrac.	0.5	—	—	—	..	—	Died during night
B. typhosus.....	Intrac.	0.25	—	—	—	..	—	Remained normal
B. coli.....	Intrac.	0.25	—	—	—	..	—	Increased irritability and recovery

died within 24 hours from overwhelming infection. Of the animals inoculated with dysentery, typhoid, and colon bacilli, all showed subnormal temperature and marked increase in irritability with occasional spastic paralysis. This is not in harmony with Bull's results, who noted poliomyelitis-like symptoms after intracerebral inoculation of *B. dysenteriae* in rabbits.

ANATOMIC AND BACTERIOLOGIC EXAMINATION

Anatomic.—There were no marked changes observed in the body cavities of rabbits dying after either intravenous or intracerebral inoculations. The spleen was rather small and gray. Occasionally a few small areas of bronchopneumonia were noticed. Microscopically there were usually some fatty changes in the liver. The nervous system showed the most marked changes. Following intravenous inoculation none of the rabbits showed any evidence of purulent meningitis. The meninges were injected but there was no marked increase in fluid. There was apparently an increase in neuroglia and round cells in both the brain and cord. Figure 8 shows an apparent increase in round cells in the anterior part of the brain. After careful comparison with normal tissues there did not seem to be much perivascular infiltration of round cells in the intravenously inoculated animals, at least nothing like what was observed after intracerebral inoculation. There was, however, a noticeable increase in round cells and neuroglia cells with apparent degeneration of ganglion cells. In the intracerebrally inoculated animals there was always, as expected, a marked cellular reaction about the point of inoculation. Some polymorphonuclear leukocytes were present but the preponderance of cells were either neuroglia or round cells. This increase in round and neuroglia cells was not, however, confined to the point of inoculation; there were always areas of marked infiltration elsewhere in the brain also. Perivascular infiltration with round cells and hemorrhages were also present at other points in the brain and occasionally in the cord, decreasing downward. In one of the rabbits there was a marked meningeal exudate, and two hemorrhagic areas were found in the center of the cord.

Bacteriologic.—Blood-agar plate cultures from the heart blood and various organs were supplemented by taking one or more pieces of tissue from each organ and dropping them into dextrose broth tubes and incubating at 37 C. for several days.

If the animal died within 24 hours after intravenous inoculation the organisms were obtained always in pure culture from heart blood, various organs, and from the brain and cord. In 3 rabbits examined 4 days after inoculation, the heart blood and organs were sterile but the brain and cord yielded pure cultures of the organisms. In a third rabbit that died 4 days after inoculation the heart blood and organ cultures from liver and spleen were sterile but a piece of heart tissue after 3 days' incubation in dextrose broth, yielded a pure culture of the organism injected. From the brain and cord of this rabbit pure cultures were obtained. In this animal the heart blood drawn just before death as well as after death, gave no growth in dextrose broth. In stained sections of the nervous system of these rabbits streptococci were demonstrated.

In the intracerebrally inoculated animals the heart blood and organ cultures were uniformly negative but pure cultures were obtained from the brain and various parts of the cord. These animals had all been inoculated in the posterior part of the cerebrum.

IMMUNOLOGICAL

We have undertaken to find answers for the following questions:

Are there agglutinins for *Streptococcus salivarius* and *mitis* in the blood streams of supposedly normal individuals?

Are there normal complement fixing bodies in the blood stream of supposedly normal individuals?

Do inoculated rabbits develop agglutinins for any of these pleomorphic streptococci?

Are these pleomorphic streptococci agglutinated by serum that Rosenow obtained by immunizing horses against pleomorphic streptococci?

We found that growth from blood-agar plates was too dry and coherent to give a satisfactory emulsion when washed off in salt solution. Fresh serum dextrose agar plates yielded a more moist growth that made a uniform emulsion. Some clumps might be present, but these would settle out by standing. The most satisfactory method was growing the organisms in calcium carbonate broth and preparing suspensions from this growth. The agglutination tests were quantitative as well as qualitative and each tube in the series was made to contain 0.5 c c of suspension and 0.5 c c of diluted serum. This would give an ultimate dilution twice that of the serum added. Ultimate dilutions are the ones tabulated. In Table 4 the results of tests with serums of various apparently normal individuals and various strains of *Streptococcus salivarius* are given.

TABLE 4
AGGLUTINATION OF *S. SALIVARIUS* BY NORMAL SERUM

Organism	Serum	Dilutions						
		1:10	1:20	1:40	1:60	1:100	1:150	1:200
C	A	++++	++++	++++	++++	++	--	--
C	D	++++	++	--	--	--	--	--
F	I	--	--	--	--	--	--	--
F	E	++++	++++	+	--	--	--	--
C	C	+++	++	+	--	--	--	--
A	A	++	--	--	--	--	--	--
F	K	--	--	--	--	--	--	--
F	B	--	--	--	--	--	--	--
F	A	++++	++++	++++	+++	--	--	--
H	A	--	--	--	--	--	--	--
C	B	--	--	--	--	--	--	--
C	I	--	--	--	--	--	--	--
F	F	+	--	--	--	--	--	--

It is interesting that among pleomorphic streptococci from over 300 throat cultures there was not a single strain of *Strep. mitis*. The

only mitis strain was obtained from a head wound in a boy. Table 5 gives the results of agglutination tests with normal human serum and this organism.

TABLE 5
AGGLUTINATION REACTIONS OF *S. MITIS* WITH NORMAL SERUM

Serum	Dilutions						
	1:10	1:20	1:40	1:60	1:100	1:150	1:200
I	+++	++++	+++	+++	+++	—	—
A	+++	++	—	—	—	—	—
D	—	—	—	—	—	—	—
C	++	++	—	+++	+++	—	—
B	—	—	—	—	—	—	—
E	—	—	—	—	—	—	—
G	+	—	—	—	—	—	—

We see that normal agglutinins for *S. salivarius* and *S. mitis* were present in some individuals as high as 1:100.

A rabbit was immunized against one strain of *S. salivarius* and complement fixation tests made with various streptococci. Serum from A, C, and D, 3 normal persons, whose blood possessed agglutinins was tested at the same time. Table 6 gives the results.

TABLE 6
COMPLEMENT FIXATION

Antigen	Serum			
	A	D	C	Immune Rabbit Serum
C	—	—	—	++++
G (<i>Strep. mitis</i>)	—	—	++++	++++
F	—	—	—	++++
<i>Strep. pyogenes</i>	—	++++	—	—
<i>Strep. viridans</i>	—	—	—	—
A	—	—	—	++++

According to the complement fixation tests *S. salivarius* and the one strain of *S. mitis* acted alike, and were sharply differentiated from the strains of *St. pyogenes* and *S. viridans*.* We found only occasional complement fixation bodies for streptococci in normal serum.

The results of tests to show whether rabbits inoculated with pleomorphic streptococci develop agglutinins are given in Table 7.

Before inoculation the blood of the rabbits was tested several times for normal agglutinations, but none were observed. We note that

* The organism was isolated from the blood stream of a patient with subacute endocarditis. On plain blood agar it produces small, nonhemolytic, decidedly green colonies. Morphologically it is not pleomorphic but possesses from 2-6 very small and uniform cells to each chain. Culturally, according to Holman, it is *S. salivarius*.

agglutinins against the strains used developed to 1:20 in one animal and 1:50 in the others. Other strains of *S. salivarius* were agglutinated by dilutions of 1:100.

Table 8 gives the results of agglutination tests of various strains of streptococci with Rosenow's antipoliomyelitis serum.

TABLE 7
AGGLUTINATION OF *S. SALIVARIUS* BY SERUM FROM INOCULATED RABBITS

Organism	Method of Inoculation	Dosage, C C	Condition of Animal	Organism	Results					
					1:10	1:20	1:50	1:100	1:200	1:400
F	Intravenous	10	Rise of temperature after 12 days	F	+++	++				
				L	++++	++++	++++	++++	—	—
2-2	Intracerebral	0.5	Permanent impairment of both hind legs	F	+++	++	—	—	—	
				L	++++	+++	+++	++	—	—
				2-2	++++	+++	++			

TABLE 8
AGGLUTINATION WITH ANTIPOLIOMYELITIS SERUM

Organism	Antipoliomyelitis Serum								
	1:25	1:50	1:100	1:200	1:500	1:1,000	1:1,500	1:2,000	1:3,000
F	++++	++++	++++	++++	++++	++++	+++	—	—
C	++++	++++	++++	+++	—	—	—	—	—
G	+	—	—	—	—	—	—	—	—
Strep. mitis	++++	+	—	—	—	—	—	—	—
1016	+++	++	++	—	—	—	—	—	—
1007	—	—	—	—	—	—	—	—	—
L	++++	+++	+++	++++	++	—	—	—	—
11-1	++	+	++	—	—	—	—	—	—
H	—	—	—	—	—	—	—	—	—
A	+	—	—	—	—	—	—	—	—
Strep. fecalis	—	+	+	—	—	—	—	—	—
Strep. pyogenes	+	+	..	—	—	—	—	—	—
Strep. viridans	—	—	—	—	—	—	—	—	—

It will be observed that antipoliomyelitis serum agglutinates strains of *S. salivarius* quite uniformly, but in variable dilutions. Strain F, for example, was agglutinated in dilutions of 1:1,500, whereas Strain C was agglutinated only by 1:200, and one strain of *S. mitis* was clumped by 1:50 dilution. All 3 of these strains showed ability to localize in the nervous system of young rabbits and produce changes accompanied by loss of muscle tone and rise in temperature. Strain C was more virulent for young rabbits than Strain F. The culture of *S. mitis* (G) was also more virulent than Strain F of *salivarius*.

DISCUSSION

In the experiments by Rosenow and his associates,² 85% of the animals showed loss of muscle tone and 15% flaccid paralysis. In our experiments 54% showed loss of muscle tone, 4.55% flaccid paralysis, and 22% rise in temperature only. The changes were increase in neuroglia and round cells and perivascular infiltration in the brain and cord. They mention that the streptococci lost their virulence rapidly and unless very early transplants were used the results were negative. We found this to be only partially true, as some of our highly virulent cultures gave positive results even after 12 weeks, being carried along on fresh serum agar and transplanted every 2 or 3 days. We found ascitic fluid dextrose broth to be very satisfactory, but also obtained positive results from dextrose serum agar plates. Our results seem to confirm most of the observations by Rosenow and his co-workers. However, there seems to be a difference of opinion as to the interpretation of the results. Bull⁵ occasionally obtained flaccid paralysis in rabbits with streptococci, and concluded that this depended on the virulence of the organism used, rather than the source. Negative results on inoculation of rabbits with filtrates of extracts of the brain and cord of poliomyelitis patients are explained by Rosenow as probably due to adult rather than young rabbits having been used. He, however, has not been successful in producing poliomyelitis in monkeys with filtrates of pleomorphic streptococci, and this is attributed to imperfect technic. Rosenow bases his conclusions that poliomyelitis is caused by a pleomorphic streptococcus on the following points: The streptococcus was found uniformly in poliomyelitis, especially the central nervous system and the throat; it produces loss of muscle tone, flaccid paralysis, rise in temperature, in animals, with round cell infiltration in the central nervous system; the blood of poliomyelitis patients contains agglutinins for this streptococcus; and specific immune serum seemed to cause an initial rise followed by a marked drop in temperature and improvement in the symptoms of poliomyelitis patients.

In our work we have found that pleomorphic streptococci, culturally and morphologically similar to the organisms found in poliomyelitis, have a wide distribution, being found in 20-25% of the throats of normal persons not associated with any outbreak of poliomyelitis; that these organisms are capable of producing similar conditions in

⁵ Jour. Exp. Med., 1917, 25, p. 557.

rabbits as those from poliomyelitis and apparently have a predilection for the central nervous system. Previously one of us¹ reported on what seemed like marked beneficial action of diphtheria antitoxin in tonsillitis in which pleomorphic streptococci appeared to be the etiologic factor. Following the injection there was usually an initial rise in temperature succeeded by a drop and marked improvement. These results were attributed to nonspecific protein effects. Rosenow,⁶ however, reports such striking results with antistreptococcus serum in poliomyelitis that it is difficult to exclude specific action.

Rosenow, Towne and Hess state that in experiments with many strains of streptococci other than the pleomorphic they have not been able to produce the poliomyelitis-like symptoms and the peculiar localization which they did with the latter organisms. We hope to study these questions further; at present we can say that one strain of non-pleomorphic *Streptococcus salivarius* showed the same tendency to remain localized in the central nervous system, but it only produced general weakness and rise in temperature, with no indication of loss of muscle tone or flaccid paralysis.

Since Rosenow's early work on elective localization there have been published numerous articles, some of which seemed to confirm and others to disprove his contentions. Gay⁷ does not conclude that Rosenow's results have been discredited and thus his positive results should take precedence over negative results by others.

In the work of Moody⁸ and of Detweiler and Maitland⁹ no attention was paid to the particular strains of streptococci other than to use organisms that might be called members of the viridans group. Krumweide and Valentine¹⁰ have shown that the viridans group is a decidedly heterogeneous one.

Henrici's¹¹ work, however, was planned with the aim of comparing the tendency of specific organisms to localize. So also was the work of Rothschild and Thalhimer¹² on *S. mitis*. Unfortunately, in Henrici's work the central nervous system was not examined thoroughly, but he mentions certain interesting nervous systems. He concludes that fermentation tests are of no significance from the standpoint of virulence

⁶ Jour. Infect. Dis., 1918, 22, p. 379.

⁷ Jour. Lab. and Clin. Med., 1918, 3, p. 721.

⁸ Jour. Infect. Dis., 1916, 19, p. 515.

⁹ Jour. Exper. Med., 1918, 27, p. 37.

¹⁰ Jour. Infect. Dis., 1916, 19, p. 760.

¹¹ Jour. Infect. Dis., 1916, 19, p. 572.

¹² Jour. Exper. Med., 1913, 19, p. 429.

or tissue localization. The work of Thalhimer and Rothschild was confined to a study of involvement of the heart by *S. mitis*.

Detweiler and Maitland⁹ apparently assume that it is unnecessary to try to classify the various streptococci used other than to group them as *S. viridans*. They cite Henrici's results in justification, but in view of Henrici's failure to include data on localization in the nervous system it is perhaps unwarranted to make such comparisons.

In regard to the tendency to localization in the nervous system observed by us, we venture to suggest that these strains of *S. salivarius* are not highly virulent in that they do not possess marked invasive powers; when injected into the blood stream in sufficiently large doses, the mechanisms for ridding the blood stream of bacteria is not able to remove all before some reach the nervous system. When once in the nervous system in sufficient number the organisms multiply because of small tendency for phagocytosis by fixed tissue cells, because the cellular reaction is mononuclear rather than polymorphonuclear and hence little phagocytosis occurs from mobile cells, and further because the medium is favorable for growth.

This explanation is borne out by the fact that when injected directly into the brain tissue the organisms do not invade the general circulation but spread throughout the nervous system only. It is possible that they are taken into the general circulation, but if they are they seem to be rapidly disposed of by phagocytic cells and were not found except when the animal succumbs to overwhelming doses within a few hours after injection.

We do not mean to say that no strains of *S. salivarius* will localize elsewhere than in the nervous system and occasionally in the heart; we only say that of the animals examined 48 hours or more after inoculation all showed localization in the nervous system and one also in the myocardium.

It is possible that there is a direct relationship between the globoid organisms described by Flexner and Noguchi¹³ in poliomyelitis and pleomorphic streptococci. If so it would be of interest that organisms with similar effects on rabbits are so widely distributed as indicated by our results.

SUMMARY

We found pleomorphic streptococci which according to Holman would be classed as *S. salivarius* in the nasopharynx of approximately 25% of apparently normal persons.

¹³ Jour. Exper. Med., 1912, 18, p. 411.

These organisms as a rule showed some pathogenicity for young rabbits, which develop agglutinins for the strains of organisms injected.

Injected intracerebrally, the cocci remained localized in the nervous system throughout which they became disseminated.

When injected intravenously they were found in the nervous system within 3 or 4 hours. After 72 hours they were present usually only in the nervous system.

The common symptoms were rise in temperature and loss of muscle tone, with occasional flaccid paralysis and permanent impairment of the legs.

The changes in the nervous system were hyperemia, frequently hemorrhage, increase in round and neuroglia cells and perivascular infiltration of round cells.

The serum of normal persons frequently contains agglutinins for these organisms, occasionally in dilutions of 1:100.

The agglutinin titer of the serum of horses which Rosenow injected with streptococci from poliomyelitis was found to be the same for the streptococci we studied as for the poliomyelitis cocci.

EXPLANATION OF PLATE

Fig. 1.—Loss of muscular power. Received 0.5 cc of a twenty-four-hour culture intracerebrally.

Fig. 2.—Marked loss of muscular power lasting more than 3 weeks.

Fig. 3.—Received 10 cc of a twenty-four hour culture intravenously; marked loss of muscle power. Permanent impairment with muscular atrophy of hind legs.

Fig. 4.—Cell infiltration near point of inoculation in posterior part of brain.

Figs. 5 and 6.—*Streptococcus salivarius* showing some of the pleomorphic characteristics.

Fig. 7.—Perivascular infiltration in anterior part of brain, point of inoculation being in posterior part.

Fig. 8.—Round cell infiltration in posterior part of cerebrum following intravenous inoculation.

PLATE



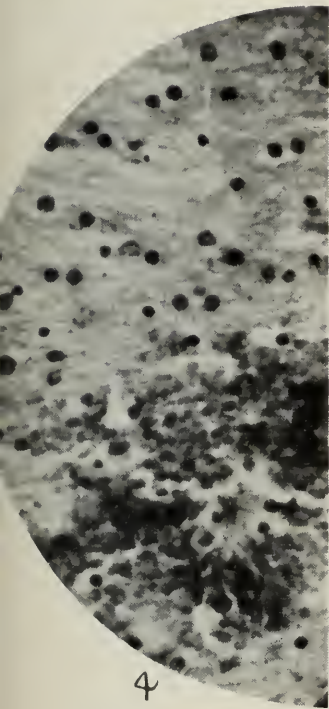
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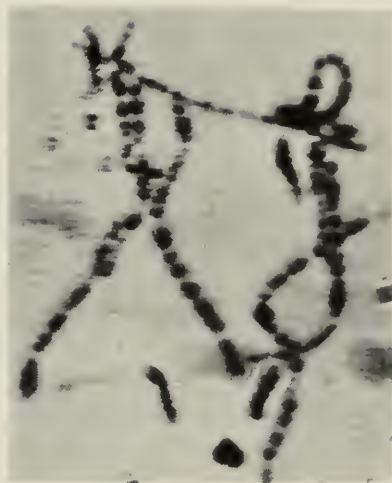
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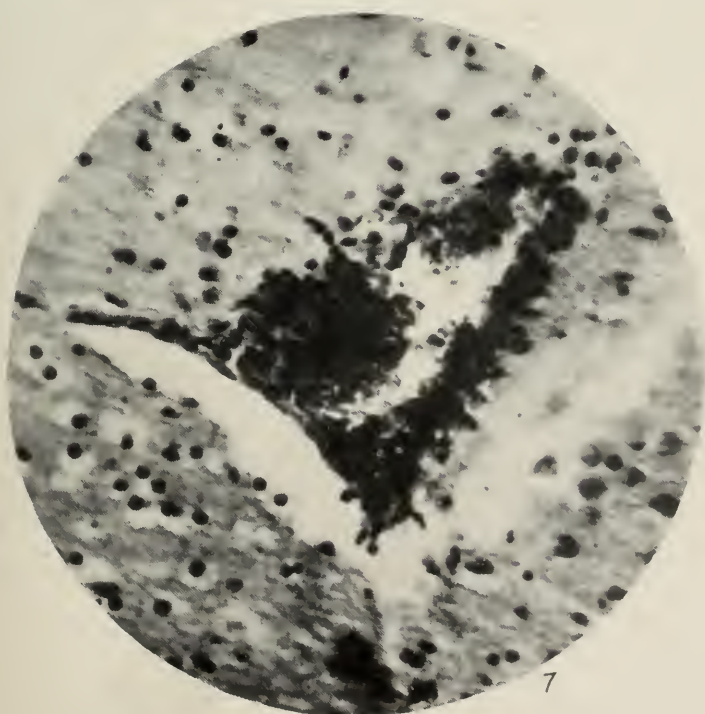
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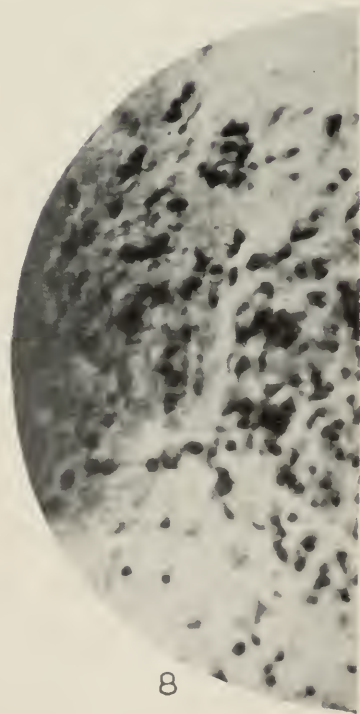
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8

VARIATION IN THE DIPHTHERIA GROUP

PERCY D. MEADER

From the Bacteriological Laboratory of Brown University, Providence, R. I.

The correct interpretation of the morphologic characters of the various organisms roughly classified under the "Diphtheria Group" has long interested bacteriologists. After the discovery of *B. hofmanni* by Hofmann-Wellenhof in 1887, many other bacilli have been reported which resemble *B. diphtheriae* so closely morphologically that many believe that these so-called pseudodiphtheria bacilli belong with the true diphtheria bacillus in one family. Some go so far as to assert that there is a transition or variation under certain conditions from the typical virulent diphtheria bacillus to the atypical nonvirulent pseudodiphtheria bacillus. A few believe that a change takes place in the opposite direction. Such theories necessarily imply that the organism which causes clinical diphtheria is subject to wide variation in its morphology as well as in its virulence. If there is variation in these characters it is natural to infer that its other biochemical characters may vary also. The present study was undertaken in an attempt to answer the following questions:

1. Do members of the diphtheria group change their morphology?
2. Do members of the diphtheria group vary in their ability to ferment carbohydrates?
3. Do members of the diphtheria group vary in their virulence?
4. Is variation in one of these characters correlated with variation in any of the others?

I. MORPHOLOGY

The morphologic resemblance of the avirulent pseudodiphtheria bacillus to the virulent diphtheria bacillus shows that if these forms belong to two distinct species, their differentiation by the microscopic examination of stained preparations is impossible. Indeed, cultures of virulent diphtheria bacilli often present distinctly different morphologic pictures. Cultures from different parts of the United States are reported to vary in their morphology.¹ Cultures obtained by me

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¹ Jour. Mass. Boards of Health, 1902,-3, 12, p. 74.

from cases of diphtheria in the epidemic at Newport, R. I., during July and August, 1917, showed large bacilli of the A A¹ B¹ C¹ type of Wesbrook's classification almost exclusively, while Providence (R. I.) cultures at the same period showed almost exclusively C¹ D¹ D² varieties. Hence the morphology of true diphtheria bacilli obtained from cases in localities very near each other may differ to a considerable degree. The granular types of the diphtheria bacillus are relatively infrequent in Providence. If the presence of granules in the diphtheria bacillus was considered necessary for a positive diagnosis, as Rickards² (1908) maintains to be the case in Boston, cultures of persons in Providence harboring virulent barred diphtheria bacilli would be reported as negative with considerable danger to the public health.

Variation in the morphology of an individual culture of *B. diphtheriae* is an entirely different matter from variation among cultures from different sources. If cultures of *B. diphtheriae* vary individually in their morphology it is of the greatest importance that this be considered in the routine laboratory diagnosis. The work of Denny³ shows that the diphtheria bacillus has a definite evolutionary cycle during a 24-hour incubation at 37 C. Wherry⁴ shows that a typical diphtheria organism may never, under certain conditions, reach a granular stage of development. The various conditions which figure in the determination of the types assumed by cultures of the diphtheria bacillus are little understood, but the chemical composition of the medium and especially its reaction may be of importance. Since dextrose, a sugar fermented by the diphtheria bacillus, is added to Loeffler's blood serum medium a constant change in reaction may be taking place as the bacteria grow. It is very improbable that exactly the same amounts of acid are produced in different parts of the same tube or in a series of tubes to which a culture may be transferred, and these variations in reaction may have some effect on type formation. It is evident that the conditions under which a pure culture of *B. diphtheriae* is growing may differ in a great variety of ways and it is a question whether these various diverse conditions do not cause a variation in type. This question is the subject of the following study.

The cultures used were obtained from 3 sources, Newport, R. I., Providence, R. I., and New York City. Cultures numbered 1 to 11 were obtained from the throats of persons infected with diphtheria during an epidemic at

² *Am. Jour. Pub. Hyg.*, 1908, 18, p. 272.

³ *Jour. Med. Research*, 1903, 9, p. 117.

⁴ *Influence of Oxygen Tension on Morphologic Variations in B. Diphtheriae*, *Jour. Infect. Dis.*, 1917, 21, p. 47.

Newport in July and August, 1917. McCoy, Bolten and Bernstein⁵ report that while this epidemic was of an "explosive" and widespread character there were very few severe cases of diphtheria.

Cultures 12-14 were obtained at the Providence City Hospital from the throats of persons infected during the epidemic at Newport.

Cultures 15-18 were obtained from cultures sent to the Providence Health Department for diagnosis. The cases of diphtheria occurring in Providence during the past year have been of unusual severity.

Cultures 19-25 were isolated from throat cultures obtained from the New York City Department of Health.

Before making any investigation with respect to variation in the morphology of the cultures they were plated on agar and colonies fished and replanted on Loeffler's serum medium. This process was repeated when there was any doubt of the absolute purity of the culture.

The following method was used in testing for variation in morphology: Each of the cultures was transferred to a fresh, moist Loeffler's serum slant and incubated 20 hours at 37 C. Permanent microscopic preparations were then made of each culture stained with Loeffler's methylene blue. The 25 cultures were then plated on dextrose blood serum agar and incubated 48 hours at 37 C. Each culture was so diluted that well isolated colonies would appear on the medium and the dilutions were so prepared that as far as possible each colony represented the progeny of a single organism. Five single colonies were fished from the plates of each culture and transferred to Loeffler's serum slants by inoculating the water of condensation and flowing it over the surface of the medium. The 125 tubes thus obtained were incubated 20 hours at 37 C. and a smear was then prepared from each tube. In making this smear care was taken to get a composite sample of the whole growth on the surface of the slant. All the types of *B. diphtheriae* occurring in each smear were recorded for future reference. One tube from the 5 prepared from each culture was selected for replating. Selection was made either because there was an approach toward a pure type culture or because types appeared which were not present in the original culture. This process of plating a selected culture and fishing 5 isolated colonies was continued through 10 successive platings when growths on blood serum from 50 colonies of each of the 25 cultures had been examined. At the end of the examination 250 permanent mounts of the selected cultures had been made while nearly 1,000 smear preparations which had not been permanently mounted had been examined for types present.

Table 1 shows the percentage of frequency of type in the progeny of the 25 original cultures. This table was derived by counting the number of times that each of Westbrook's types appeared in the tubes obtained from each of the 25 original cultures and dividing this number by the number of opportunities for each type to appear. Enough measurements of types were made and compared with the measurements reported by Westbrook⁶ (1900) to make certain that the proper letters were assigned to the types present in the cultures.

⁵ Pub. Health Rep. U. S. Mar. Hosp. Serv., 1917, 32, p. 1787.

⁶ Tr. Assn. Am. Phys., 1900, 15, p. 198.

TABLE 1
PERCENTAGE OF FREQUENCY OF TYPE IN THE PROGENY OF 25 CULTURES

Culture Number	Original Type	A	A ¹	A ²	B	B ¹	B ²	C	C ¹	C ²	D	D ¹	D ²	E ²
1	ACC ¹ DD ¹	2	2	0	8	6	0	34	51	6	57	77	53	0
2	A ¹ B ¹ CC ¹	10	6	0	8	8	0	50	65	15	50	71	34	0
3	DD ¹ D ²	6	6	4	2	8	0	40	85	29	19	61	51	0
4	C ²	0	0	2	0	0	0	0	0	100	0	0	8	0
5	DD ¹ D ²	2	2	0	0	4	0	35	60	12	43	84	67	0
6	C ¹ D ¹ D ²	4	2	2	0	0	0	42	85	19	38	76	57	0
7	AC ¹ C ² D ²	6	0	2	0	0	0	6	45	87	2	18	14	0
8	C ¹ D ¹ D ²	0	0	0	0	0	0	26	34	2	38	85	85	0
9	C ² D ²	0	0	0	0	0	0	0	4	0	15	57	95	0
10	C ¹ D ¹ D ²	2	2	0	2	2	0	57	73	12	59	67	44	0
11	C ¹ D ¹ D ²	0	2	0	0	0	0	12	44	16	8	93	87	0
12	D ²	0	0	0	0	0	0	0	0	0	0	0	83	16
13	B ¹ C ¹ C ² D ²	0	0	0	0	0	0	0	6	10	0	18	91	4
14	A ¹ B ¹ C ¹ D ¹	10	2	0	0	8	0	18	85	37	18	77	66	0
15	CC ¹ DD ¹	0	0	0	0	0	0	6	41	18	12	91	85	0
16	D ¹ D ²	8	0	0	2	2	0	21	73	21	21	88	65	0
17	AA ¹ C ¹ D ¹	8	0	0	0	0	0	6	55	21	17	82	87	0
18	C ¹ C ² D ¹ D ²	0	2	0	0	0	0	6	61	72	6	51	68	0
19	D ¹ D ²	0	0	0	0	0	0	0	0	2	0	44	100	0
20	D ²	0	0	0	0	0	0	0	6	8	0	54	100	0
21	D ¹ D ²	0	0	0	0	0	0	0	12	4	0	43	100	0
22	D ¹ D ²	0	0	0	0	0	0	0	8	2	0	40	97	0
23	D ¹ D ²	0	0	0	0	0	0	0	0	0	0	17	100	0
24	C ¹ DD ¹ D ²	6	0	0	2	0	0	46	79	14	40	85	59	0
25	D ²	0	0	0	0	0	0	0	0	6	0	6	100	0

Several interesting facts are shown by Table 1. The various forms of the A and B types are relatively infrequent in the progeny of the 25 original cultures even when an A or B type was present in the culture used for the first plating. Types of the C and D groups are the ones which predominate over all others while E² was the only representative of the smaller forms of B. diphtheriae which appeared in any of the series of cultures. Cultures 3, 5, 16, 19, 20, 21 and 25 indicate that although a culture may show only forms of the D group of Wesbrook's classification, its progeny may develop forms of the C group. Cultures 3, 5 and 16 produced a large number of the C group forms while the others showed only a few. Another surprising fact brought out by the table is that cultures of the solid staining C² and D² types may show in later growth barred and even granular types in greater or less abundance. Cultures 9, 20 and 25 produced various forms of the C and D groups which were not present in the original culture and which would not be expected to occur in a pure culture of a solid staining type. Cultures 13, 21, 22 and 23 showed a gradual replacement of the barred types by the solid staining types.

A fair definition of variation in type would be that the predominating types present in subcultures were different from the types present

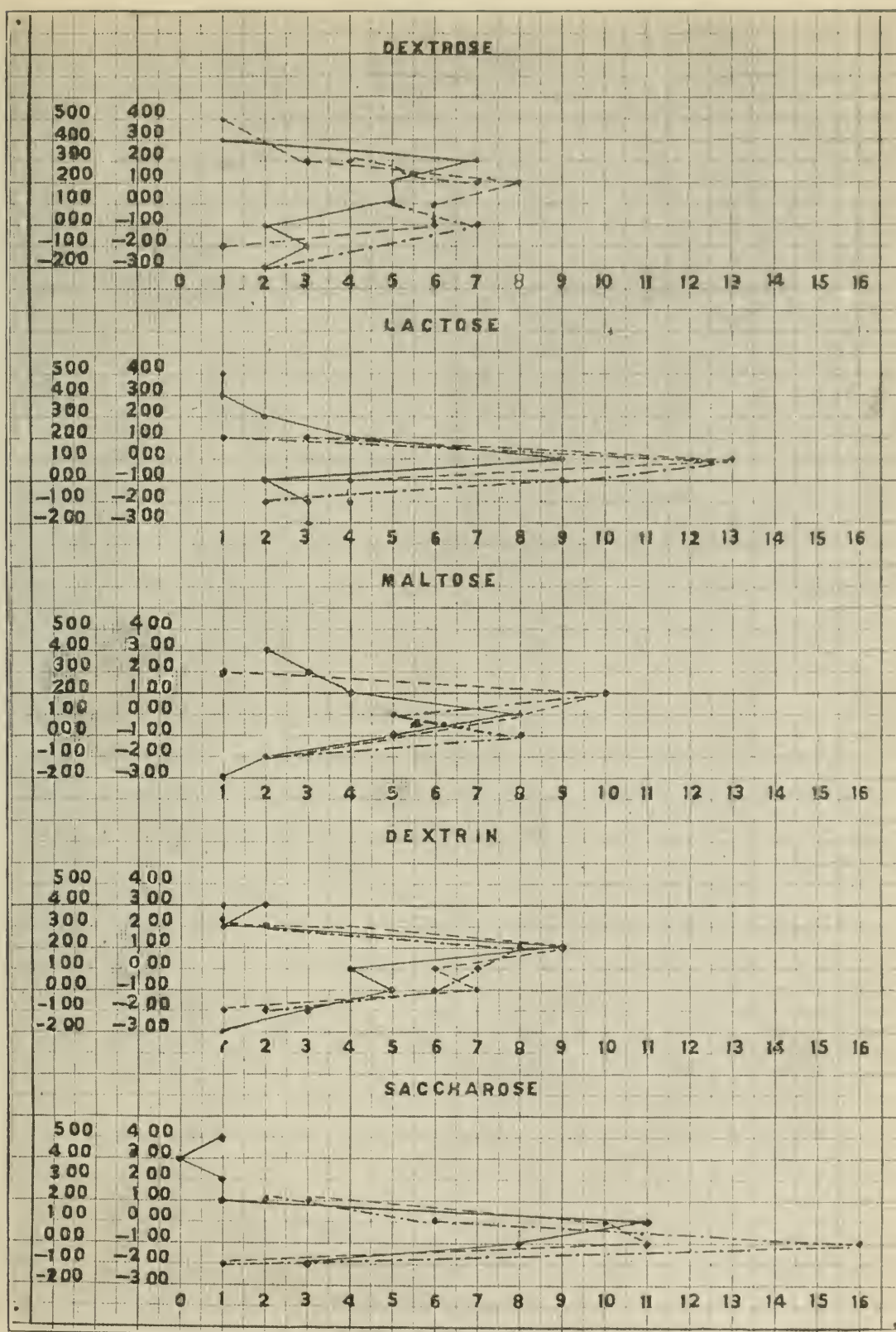


Chart 1.—Frequency curves of fermentative reactions of the 25 original cultures and after the fifth and tenth platings.

in the original culture. Williams⁷ using this criterion found that none of the cultures examined had varied. The types seemed rather to revolve around a mean so that transfers from each culture under examination showed types characteristic of the first culture examined.

Using this criterion of variation as a standard a study of Table 1 shows that some of the cultures have varied while others have not. Cultures 1, 4, 6, 7, 8, 11, 12, 18, 19, 20, 21, 22 and 25 show enough of the original types appearing in the later tubes to justify the statement that they have remained reasonably true to type even after 10 platings. Cultures 2, 3, 5, 9, 10, 13, 16 and 17 show by the percentage of types present in the subcultures examined that a distinct variation in type has resulted from the selection of different colonies for replating. There is reasonable doubt whether cultures 14, 15, 23 and 24 show real variation. It appears from this analysis of the results of the experiment described above that 8 members of the diphtheria group have shown morphologic variation, 4 may have varied only slightly, if at all, while 13 showed no reasonable indication of variation. It would seem that some cultures have the ability to vary in morphology while others lack this characteristic.

II. STUDY OF FERMENTATION

The power of bacteria to break down carbohydrates with the production of acid is in many cases a valuable means of classification. We are by no means certain, however, that the testing of the fermentative reactions of members of the diphtheria group has any real value for diagnosis. To be of diagnostic value the test must separate the virulent diphtheria bacilli from those true diphtheria bacilli which have lost their virulence as well as from the so-called "pseudodiphtheria" organisms. A survey of the work done by different investigators would lead one to conclude that fermentative reactions are of practically no value for diagnosis. Fermentative reactions may, however, be of considerable value in classifying the group.

Knapp⁸ and Zinsser⁹ found that *B. diphtheriae* fermented dextrose, maltose and dextrin, but not saccharose, while *B. xerosis* fermented dextrose, maltose and saccharose, but not dextrin. *B. hofmanni* failed to ferment any of the carbohydrates. Moshage and Kolmer¹⁰ found that fermentative reactions were too irregular to be of use in determining the virulence of a culture of diphtheria bacilli. The following

⁷ Jour. Med. Research, 1902, 8, p. 83.

⁸ Jour. Med. Research, 1904, 12, p. 475.

⁹ Jour. Med. Research, 1907, 17, p. 277.

¹⁰ Jour. Infect. Dis., 1916, 19, p. 19.

study was undertaken to determine whether subcultures varied in their fermentative power from the cultures from which they were obtained.

The 25 cultures described in the previous section of this study were used and fermentation reactions were tested before the first plating, after the fifth and after the tenth platings on each of the cultures. Sugar-free nutrient broth prepared according to the Standard Methods of the American Public Health Association (1912) was used to which was added 1% of the various carbohydrates (Merck's). The medium was sterilized at 15 lbs. pressure for 15 minutes since Mudge¹¹ has shown that sugar mediums so sterilized are hydrolyzed less than when sterilized in the Arnold. All fermentation tests were made in triplicate and 3 control tubes were incubated and tested with each set of tubes. The period of incubation at 37 C. for maximum fermentation with the carbohydrates used in this study was determined as Morse¹² had previously done. It was found that the maximum acidity was produced in dextrin broth on the 8th day, in saccharose broth on the 9th day, in dextrose and maltose broth on the 12th day and in lactose broth on the 14th day of incubation. Throughout the study of fermentation tests to be described titrations were made on the days indicated by this preliminary test. Five cc amounts of the cultures to be tested were titrated according to the Standard Methods of the American Public Health Association using phenolphthalein as an indicator. All titrations were properly checked with control tubes of sterile broth which had been incubated an equal length of time.

TABLE 2

THE COMPARATIVE REACTIONS OF 25 CULTURES AND THEIR DESCENDANTS AFTER THE FIFTH AND TENTH PLATINGS

Culture Number	Dextrose			Lactose			Maltose			Dextrin			Saccharose		
	0	5	10	0	5	10	0	5	10	0	5	10	0	5	10
1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
3	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
4	+	—	—	+	—	—	+	—	—	+	—	—	+	—	—
5	+	+	+	+	+	+	+	+	+	+	+	+	+	—	—
6	+	+	+	+	+	+	+	+	+	+	+	+	+	—	—
7	+	—	+	+	+	+	+	—	+	+	+	+	+	+	+
8	+	—	+	+	—	+	+	—	+	+	—	+	+	—	—
9	+	+	+	—	+	—	+	+	+	+	+	—	+	—	—
10	+	+	+	+	—	+	+	+	+	+	+	+	+	—	—
11	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
12	+	+	+	—	—	+	+	+	+	—	+	—	+	—	—
13	—	—	—	—	—	—	—	—	—	—	—	—	+	+	+
14	+	+	+	+	+	+	+	+	+	+	+	+	—	—	+
15	+	+	+	+	+	+	+	+	+	+	+	+	+	—	+
16	+	+	+	+	+	+	+	+	+	+	+	+	—	+	+
17	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
18	—	—	+	+	—	+	—	—	+	—	—	+	—	—	+
19	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
20	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
21	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
22	—	—	+	—	—	+	—	—	+	—	—	+	—	—	+
23	+	—	—	—	—	—	—	—	—	—	—	—	—	—	—
24	+	+	+	+	+	+	+	+	+	+	+	+	—	—	—
25	—	+	—	—	+	—	—	+	—	—	+	—	—	—	—

Explanation: 0 = The original cultures; 5 = After the fifth plating; 10 = After the tenth plating.

¹¹ Jour. Bacteriol., 1917, 2, p. 403.

¹² Jour. Infect. Dis., 1912, 10, p. 253.

Table 2 shows the comparative fermentative reactions of the 25 cultures. Four of these cultures, 1, 8, 10 and 17, are virulent diphtheria bacilli which ferment not only dextrose, maltose and dextrin, but lactose and saccharose also, although the first three vary in this respect. Indeed, it is worthy of note that the fermentative reactions as investigated at 3 different stages of cultivation of these 25 cultures vary in many cases. Cultures 1, 2, 13, 17, 19, 20, 21 and 24 are the only ones whose titrations remained constant throughout the experiments. Table 3 gives the Wesbrook types present in the cultures at each titration together with their virulence and the types recovered at necropsy. Chart 1 shows further details of the fermentation tests while Chart 2 shows frequency curves derived from all the tests shown in Chart 1.

This study demonstrates that more than half the cultures investigated varied after successive platings in their power to produce acid from carbohydrates. Fermentation tests are of no value in testing virulence since even virulent cultures do not remain constant in their fermentative power. The frequency curves of Chart 2 indicate that virulent and nonvirulent diphtheria cultures as well as those members of the pseudodiphtheria group included in this study belong to one large group of organisms whose fermentative reactions vary around a single mean.

III. STUDY OF VIRULENCE

The control of the spread of diphtheria has become largely a matter of recognizing "carriers" of diphtheria bacilli by a demonstration of the presence of diphtheria organisms in the nose or throat. These bacilli are identified by their morphology, and conclusions in regard to their virulence are frequently based largely on the particular types of bacilli which are found. The relation of virulence to morphology is therefore of great importance.

There is general agreement that different strains of *B. diphtheriae* vary in their virulence for guinea-pigs, but there is considerable disagreement whether an individual culture can vary in virulence to any great degree. Rickards¹³ reports finding that virulence tests made from different colonies of the same culture varied. He leaves the cause of this variation in virulence an open question, although he states that the cause was not a variation in the resistance of the guinea-pigs used.

¹³ *Am. Jour. Pub. Hyg.*, 1908, 18, p. 292.

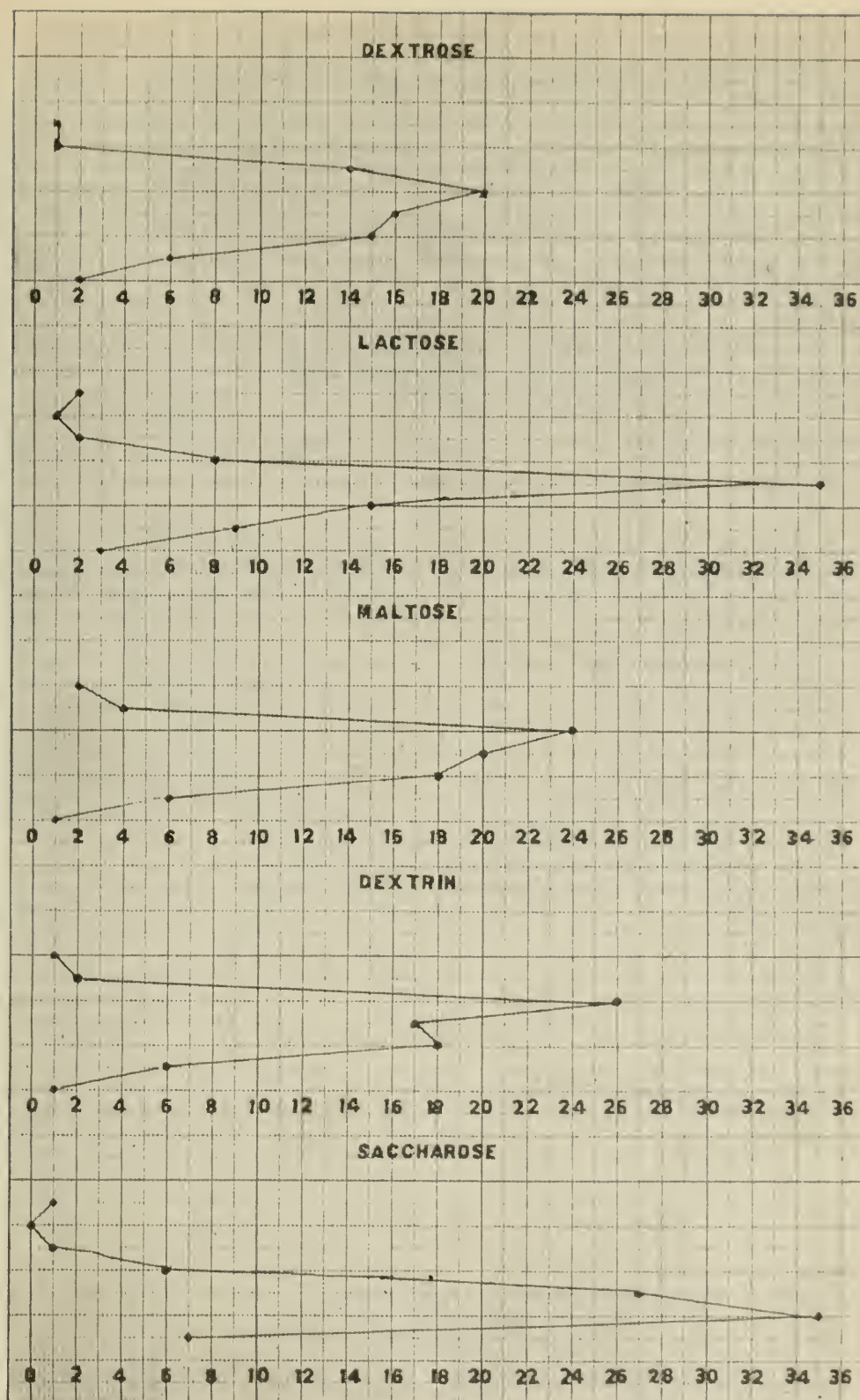


Chart 2.—Frequency curves of all 75 cultures shown in Chart 2.
 Fermentative reactions of original cultures = ————.
 Reactions of cultures selected after the fifth plating = — . — . — . — .
 Reactions of cultures selected after the tenth plating = — — — — — — — — — —.

The epidemic of diphtheria in Newport, R. I., in the summer of 1917 well illustrates the fact that different strains of widely different degrees of virulence may occur in localities relatively close to each other. McCoy, Bolten and Bernstein⁵ report that of 402 cases occurring in Newport there was only one death, while in Providence, R. I., there were during the same period 8 fatal cases among 62 reported, a case death rate of 12.90.

The difference of opinion in regard to variation in the virulence of the diphtheria bacillus and the importance of this question in the control of the disease led to the following study to attempt to determine whether the progeny of pure cultures of members of the diphtheria group vary in virulence from the parent culture and from each other.

The cultures used in this study were the same as those used in the studies on morphology and fermentative reactions. Virulence tests were made on the original cultures, the cultures selected after the fifth and after the tenth platings. Virulence tests were made by intraperitoneal inoculations of the culture grown in broth for 24 hours at 37 C. using guinea-pigs of weights varying from 200-350 gm. An amount of culture corresponding to 1% of the body weight of the animal was used for inoculation. The weight of the animals before inoculation and the daily weights for a period of 7 days following the inoculations were recorded. All pigs that died were examined at necropsy and material for cultures was taken from the principal organs and fluids. The types of organisms recovered at necropsy were compared with the types inoculated.

Table 3 shows the virulence of the 25 original cultures and their virulence after the fifth and tenth platings. Cultures 16, 17 and 24 were the only ones which proved virulent in each of the 3 tests. Five cultures proved virulent in 2 of the 3 tests. Of these, Cultures 5 and 6 are of peculiar interest because, although the original cultures were nonvirulent, the cultures selected after the fifth and tenth platings in each case proved virulent. Four cultures were virulent in 1 of the 3 tests, while the remaining cultures were consistently nonvirulent.

A comparison of the types inoculated with those recovered at necropsy shows that there is wide variation, a phenomenon which has been noted by other investigators. The types in Culture 5 after the fifth plating were B² C² D², yet when this culture was recovered at necropsy it showed ACC¹ DD¹ types. On the other hand, Culture 6, after the tenth plating contained CDD² types, but on recovery at necropsy C²D² types occurred. Again, Culture 24, after the tenth plating contained only solid staining organisms when recovered at necropsy, although C¹ D¹ D² types were inoculated.

A study of the relation of types inoculated to virulence brings out certain facts. Cultures containing only barred and solid staining types proved virulent, and in the case of the strain selected after the fifth plating of Culture 5 a culture containing only solid staining types proved virulent. While the most frequent morphologic picture of Culture 5 showed a mixture of granular, barred and solid staining types, the fact remains that at this particular stage of its cultivation solid staining forms only were present. It is also of interest that cultures containing granular forms were frequently nonvirulent. Those cultures which consisted of solid staining forms for the greater part of their cultivation were consistently nonvirulent.

In order to determine whether the results obtained in those virulence tests were due to variation in the virulence of the strains injected or to variation in the resistance of the guinea-pigs a further set of inoculations was made. Five pigs from different caviaries, of as nearly the same weight as possible, were inoculated with a broth culture of Number 1, selected after the fifth plating. This culture was grown in a large Erlenmeyer flask, and all the pigs were inoculated under as nearly the same conditions as possible. The original strain of Culture 1 and the strain selected after the tenth plating were virulent, while the strain selected after the fifth plating proved nonvirulent. This experiment should prove the truth of the first set of inoculations. Since none of the 5 pigs inoculated died it is evident that the culture varied in virulence rather than that the guinea-pigs varied in resistance.

This study shows that subcultures from a pure culture of members of the diphtheria group vary in virulence from the parent strain. The morphology of a virulent culture is often altered by passage through a guinea-pig. Granular and barred types are usually virulent, while solid staining types which retain this morphology through many generations are nonvirulent. Morphology is not, however, an index of virulence.

CONCLUSIONS

From this study of 25 cultures of members of the diphtheria group isolated from various sources the following conclusions appear justified:

From a biometric study of the fermentative reactions of members of the so-called diphtheria group it appears that they constitute a genetically related group of organisms.

Variations in morphology occur in subcultures derived from one parent strain.

Variations in fermentative reactions occur in subcultures derived from one parent strain.

Variations in virulence occur in subcultures derived from one parent strain.

The virulence of a strain is not closely correlated with its morphology.

The virulence of a strain is not correlated with its fermentative reactions.

A BACTERIOLOGIC STUDY OF SARDINES *

MAUD MASON OBST

The yearly loss from the swelling of cans of sardines has been stated by the canners to be 1% or higher, and in some cases it has been reported to be 30% of the output. Prescott and Underwood,¹ in the study of the canning of clams and lobsters, found several species of living bacteria in the swelled goods. Cathcart² isolated 4 organisms from canned sardines. These included *B. coli*, 2 organisms which were pathogenic to guinea-pigs when injected intraperitoneally and 1 nonpathogenic. In 1913, during a preliminary investigation of the canning of clams in an unpublished account, Dr. Payn B. Parsons³ reported the finding of gas-producing facultative anaerobic bacteria in the commercially processed goods. He found it necessary to process the clams at 240 F. in order to insure complete sterilization. This fact, of course, suggested the presence of bacterial spores, but they were not demonstrated culturally, nor was their relation to oxygen definitely determined. It was, therefore, considered probable that such organisms might be the cause of the swelled cans of sardines. It seemed possible also that the organisms which caused the trouble in clams might be identical.

A number of swelled cans of sardines received from packers at various places along the coast of Maine, including Eastport, Lubec, North Lubec, Machiasport, Jonesport, Boothbay Harbor, and Rockland were examined for both aerobic and anaerobic bacteria. The cans were carefully sterilized on the outside by flaming with alcohol and opened with a sterilized can opener. In many cases when the cans appeared badly swollen their contents were entirely decomposed, all connective tissue and even the bone tissue of the sardines having been destroyed until no resemblance of a fish form remained. The contents were reduced to a soft pasty mass full of gas bubbles and this mass emitted a disagreeable odor of sourness and hydrogen sulphid.

Aerobic plate cultures were made from several cans on fish, dextrose, and nutrient agar and in dextrose and nutrient broth. In practically no instance were any living aerobic bacteria found, except in those cans which had been burst by swelling. From each of the 287 cans examined, which were not burst,

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* This article deals with the bacteriologic results which were obtained during the studies conducted in the sardine industry in collaboration with the Animal Physiological Laboratory of the Bureau of Chemistry, Washington.

¹ *Technology Quarterly*, 1897, 10, p. 183.

² *Proc. Bact.*, 1906, 6, p. 248.

³ *Bacteriologist*, Bureau of Chemistry, Washington, D. C.

an anaerobic organism was found in pure culture. This organism was obtained only in its spore stage. Morphologically and in many of its cultural characteristics it closely resembles the bacillus of symptomatic anthrax (*B. rauschbrandi*). It, however, is probably more nearly related to the organism described by Ivar Nielsen⁴ as *Bacillus walfischrauschbrandi* and will thus be designated hereinafter. Its interesting characteristics are its ability to form gas from protein or carbohydrate mediums, and the rapidity with which it forms spores. When grown under laboratory conditions, no vegetative cells without spores were found in 8-hour old cultures. It is hoped that the method of cell division of this organism may be studied further. Only 10 cans of imported (Norwegian) sardines have been examined, and of these, 7 which were appreciably swollen, contained *B. walfischrauschbrandi*.

In cannery practice the sealed cans of sardines are heated in an open bath of boiling water. The time of processing varies in different factories from 1¼-2½ hours. The water is usually heated either by free steam entering at the bottom or by steam coils. The tanks in which the following experiments were conducted were about 4 feet long, 2½ feet wide, and 4 feet deep, and held about 65 cases of cans. Cans of sardines were inoculated with *B. walfischrauschbrandi* and placed in various parts of the boiling tanks. Of 11 cans placed near the surface 7 were processed 1½ hours and the remainder for 2½ hours. The *B. walfischrauschbrandi* spores were recovered from 5 of the 7 cans while cultures from only 1 of the 4 which were heated for 2½ hours showed growth. Of the other cans, only 1 showed living organisms after processing.

Temperatures were taken of the contents of cans placed well beneath the surface of the water in the boiling tanks by means of a thermocouple attached to a potentiometer. It was found that in one case 12 minutes were required to heat the interior of the can from 45-99 C. In another attempt, 25 minutes' boiling raised the temperature from 36-100 C. If 25 minutes are taken from 75 minutes, the time of actually heating cans in some factories, it does not allow a very great margin over the time absolutely necessary for killing the spores of the bacteria. In many instances, too, cans are allowed to float in the water or remain only partially covered with water. This prevents thorough processing. Cans, however, which are completely filled without leaving any air space and which do not leak, do not float. Their own weight holds them beneath the water.

An experiment was made to determine if bacteria lived through the process of frying in an oil bath at a temperature of 240 F. It was found that *B. walfischrauschbrandi* planted in the meaty portion of a herring⁵ which was protected by some portion of other herring both above and below was not killed. In one instance a stomach containing feed was found in a little heap of three fish which overlapped one another, and from it was isolated a bacillus which is described later in this paper as *Bacillus B.*

In an attempt to learn from what source *B. walfischrauschbrandi* found its way into the cans, moist, greasy dirt was scraped from the inside of the pickling tanks of the sardine canneries and cultures prepared. The spores of this organism were present in large numbers. They were also obtained from dirt on the floors beneath the flaking machines in certain factories. Since it has been impossible to demonstrate the presence of the vegetative

⁴ Centralbl. f. Bakteriöl., 1880, 7, p. 267; Kitt: Th. Bacterienkunde, 1903, p. 301.

⁵ "Herring" is used throughout this paper to designate the sea herring or *Clupea harengus* which is used for sardines along the Maine coast.

form of the bacillus even in the laboratory mediums, it could not be definitely determined whether multiplication takes place under the conditions where it has been found or whether it is present in the spore form only. While searching for *B. walfischrauschbrand* in the factory, it was found that *B. welchii*, also a spore-forming, gas-producing bacillus, entered 3 of the factories through the water supply used for washing and pickling the herring. The presence of *B. welchii* indicates sewage, although it gives no clue to the recentness of the deposit. In one instance its source was located in the mud directly beneath the opening of the intake water pipe. The end of this pipe was about 2 feet above the mud at the time of sampling. The shore was sloping at this point and the mud showed evidences of having been recently dug up by the prow of a boat. Any boat coming into the region at low water disturbed the water and caused that which entered the factory to show a large amount of sediment. In another instance the intake pipe to the water supply for washing and pickling the fish had its opening directly on the gravel bottom of the shore about 15 feet from the outlet of one of the main sewers of the town. It was thus clear that dirt or filth in the factories might be one source of pollution in the product.

These organisms were next sought in and on the fish as they came to the factories. In November, 1915, herring were taken alive from a weir near Boothbay Harbor, Maine, in sterile containers. Sterilized glass fruit jars, which were kept sterile on the outside by being tied in cotton bags before being autoclaved were used for containers. After a little practice the live herring were readily dipped with the sterile jars directly out of the seine before they were lifted from the water. At the laboratory the herring were dissected with sterile instruments, and sections placed in sterile culture tubes. Dextrose agar was then poured into the tubes and allowed to solidify. This was found to provide an anaerobic condition which permitted the growth of strict and facultative anaerobes. Tube and aerobic plate cultures were made at first with both dextrose and fish agar, but as there appeared to be little advantage in using the fish agar for tube cultures it was omitted.

The flesh of the herring fresh from the water was aseptically removed by holding the fish with sterile forceps while the skin was cut away from the thick meat on the side of the fish, and then a portion of this meat was cut out with sterile implements. To between 2.5 and 5 gm. of the flesh thus obtained was added sterile water in the ratio of 1:9. This was then shaken with sterile shot and 1 cc portions were plated on dextrose agar and fish agar. The remainder in the flask was then covered with fish agar. All were incubated at 37 C. Several fish thus examined showed the growth of a few organisms, but only 2 of 56 showed more than 100 bacteria to the gm. The majority of the plates showed from 1-5 colonies of bacteria on the 1 cc plates of the above liquid. These may have been accidental contaminations. In no instance were gas-producing bacteria present.

Sections of the stomach and intestines were aseptically removed, split lengthwise, washed with sterile water, and cultures made of the entire portions, the wash water and sections separately. In several instances special tests were made to determine the sterility of the stomach and intestines in the absence of feed. Of 94 herring thus examined in which the absence of visible feed was noted at the time of dissection, 13 only showed the presence of any bacteria in the stomach or intestines. In many instances the stomachs were free from both feed and bacteria, when partially digested feed, living bacteria, and occasionally gas were present in the intestines. The gas was usually found only after the herring had been held from 5-12 hours after removal from

the water. In those fish in which the stomachs contained feed the dorsal ends were often markedly distended with gas. *B. walfischrauschbrand* was found in three instances. No other bacteria were isolated. Three herring which contained feed in the form of tiny fish, were examined. These contained no bacteria in their stomachs. In nearly all fish which contained the feed known among the fishermen as "red feed," bacteria capable of producing gas were found. *Bacillus B.* was found either in pure culture or accompanied by an aerobic white micrococcus in the digestive organs of many herring which contained copepods. Where the schizopod eyes were present in the stomachs of herring, *B. walfischrauschbrand* was frequently found, in several instances alone, yet often accompanied by *Bacillus B.* From this work it is suggested that the very simple digestive tract of the herring mechanically frees itself of bacteria shortly after all feed is eliminated (Plate I).

The gills of the herring were almost always found to harbor living bacteria whenever they were present in any part of the fish and in about 40 herring of which all other portions were sterile. *Bacillus B.* was isolated in only one instance from the gills, but *B. walfischrauschbrand* was found in all others which showed gas-producing organisms. It seems probable that the gills may filter the spores of the latter bacillus from the water which passes through and hold them in the spore stage. The herring from which *B. walfischrauschbrand* was isolated were obtained from the following places:

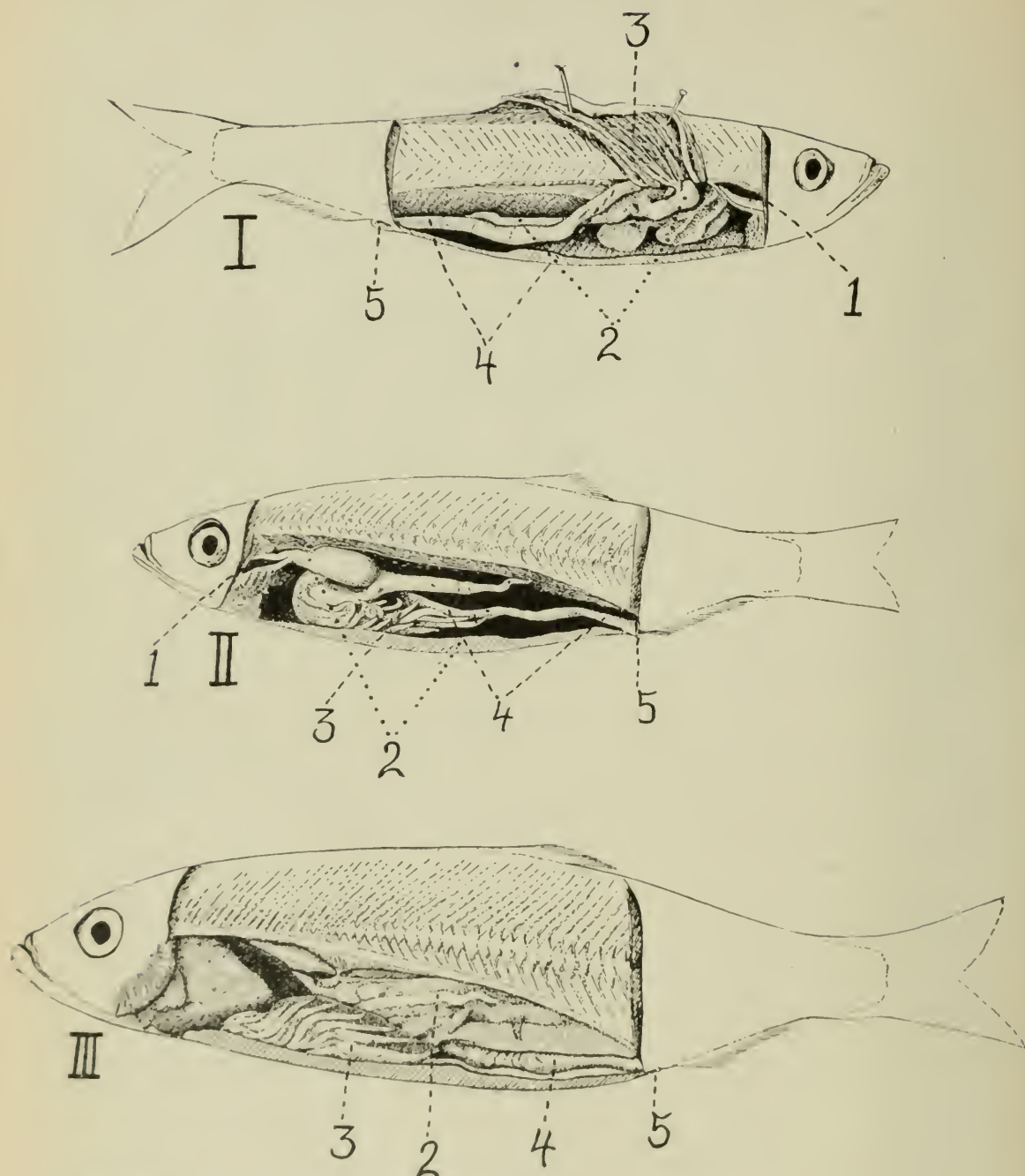
1. Weir near Boothbay Harbor, Maine.
2. Weir at Hog Island, South Bay, near North Lubec, Maine.
3. Seine at Denbrow Pt., Cobscook Bay, near North Lubec, Maine.
4. Weir at L'Etang Harbor, near L'Etang Peninsula, N. B. Canada.
5. Boat at Machiasport Wharf, Machiasport, Maine.

Bacillus B. was found in fish from the third and fourth places mentioned, and also in fish obtained from boats in the harbor at Eastport, Maine, probably brought from Irish Channel, near Deer Island, Canada, and from Back Bay, Canada. It was obtained in herring taken direct from a weir near Three Islands off the southeast coast of Grand Manan Island, Canada.

From several herring the stomach contents, comprised apparently entirely of copepods, were removed and incubated in a tube with no added mediums under anaerobic conditions. Gas accompanied by a foul odor was present after 5 hours' incubation at 37 C. *Bacillus B.* was isolated. It may be noted that under the description of *Bacillus B.* it was found to produce gas rapidly in the presence of horse blood in sugar mediums.

From this work it seemed evident that *B. walfischrauschbrand* and *Bacillus B.* might accompany the feed of the herring, especially the schizopods and copepods while living, and on this feed be carried into the digestive tract and thus be brought to the factory. These forms of feed were therefore collected fresh from the water and examinations made.

The schizopods were found in large numbers in the water around Wilson's Beach, Campobello Island, in the cove on Moose Island above Dog Island; around the wharves at Eastport and Lubec, and in smaller numbers in the southern part of South Bay. These waters with the exception of South Bay all contain waste products discarded from fresh fish trimmings or from various factories, and the portion of South Bay where the schizopods were found is rich in organic matter and does not receive much purification by the tide. The food supply probably partially influences the location of the schizopods and, doubtless, their bacterial associates. It seemed significant that in regions where



1. Mouth.
2. Stomach.
3. Appendices to the dorsal end of the stomach.
4. Intestine.
5. Anus.

the sardine packers and others avoided polluting the water with waste products schizopods were seldom seen. The schizopods were most easily caught at about 3 hours after high water when they were found in masses among the rocks which were covered with sea-weed. They were scooped with a net fastened into the hoop of a dip-net handle. The net used was made of a coarse mesh linen scrim sewed to a 6 inch wide band of heavy cotton cloth. The lower part of the net was left open and a gathering string sewed in so that it could be tied firmly around the neck of a glass jar. For this investigation sterile jars of the Lightning type were used and the net was autoclaved in a cotton bag in which it was kept until used. The schizopods were caught in sterile containers and kept alive in sea-water until they reached the laboratory. It was noted that when they were placed in 30% alcohol at room temperature for 24 hours the schizopods emitted a large volume of gas and disagreeable odor. The surface of the alcoholic solution was often covered with foam. The crawfish, which has a shell of calcareous chitin, will close this shell with its muscles and hold out preservatives until long after death, thus allowing the decay of the meat within the shell by bacterial action. This evidently happens in the case of the schizopods, which have a similar chitinous shell.

Anaerobic and aerobic cultures were made of the entire schizopods and of sections cut aseptically from the bodies. Not all schizopods showed bacterial growth. Many of the whole bodies and sections of the shell covering showed aerobic bacteria but they were not identified. Sections from the thoracic and digestive regions showed the presence of bacteria including *B. walfischrauschbrand*, and in two instances *Bacillus B.* The dorsal sections of the body showed no growth.

In the complete report on this subject, Dr. Weber calls attention to the care exercised by the French sardine industry in securing clean bait for catching the fish. The following is quoted from his report: "So particular are the fishermen and the manufacturers to avoid the decomposition when fish are feedy, that the strictest attention is given to the quality of the bait. In early times in this industry, the use of a prepared bait containing especially powdered prawn and shrimp was forbidden by royal decree. As late as 1853, the use of this bait was forbidden. It was held that it spoiled the fish by facilitating decomposition." If the shrimp used contained originally *B. walfischrauschbrand*, the spores of this bacillus could have resisted the process of drying and powdering by which the bait was prepared. It would then have been capable of facilitating the decomposition of the fish.

The copepods were collected in large quantities off Whale Cove between Swallow Tail Light and Bishops Rock, Grand Manan Island, Canada, and in small quantities directly east from Grand Manan, by using a copepod net,⁵ sterilized, with a sterile glass jar attached.

Individual copepods were cultured in dextrose and fish agar, and masses of the copepods were placed in large tubes covered with the above mediums, and incubated under anaerobic conditions. Abundant gas formed in the latter after 6 hours' incubation at 37 C. and *Bacillus B.* was isolated. Nearly all of the individual copepods which were incubated showed the presence of *Bacillus B.* *B. walfischrauschbrand* was obtained from the copepods in one instance, but as it was found no more it was considered that this might have been a contamination. A white facultative aerobic micrococcus was found among the copepods with *Bacillus B.* but no other organisms were isolated.

⁵ This net is one of particular design which was loaned for this work by the U. S. Bureau of Fisheries' Experiment Station, Woods Hole, Mass.

Hörhammer⁶ demonstrated the presence of clumps of bacteria within the bodies of crustacea by staining them with neutral red or methylene blue. He worked principally on cyclops but mentioned having studied other copepods.

When the herring are held in the weirs they discard feces in appreciable quantities. The feces of those herring which have fed on copepods and schizopods float near the surface of the water in pink sections about 10 cm. long by about 5 cm. in diameter. One sample of this was collected with a little water from the southwestern part of Passamaquoddy Bay, not many miles from Wilson's Beach, where schizopods were found. A culture of *B. walfischrauschbrand* was isolated from the entire sample.

TABLE 1
TEMPERATURE CHANGES IN HERRING DURING TRANSIT

Method of Treating Herring in Transit	Amount of Feed Present	Time	Temperature in Degrees Centigrade	Remarks
Floated in brine containing 150 lbs. salt per hogshead of fish	Slight	6:30 a. m.	12	At weir
		7:00 a. m.	13	
		7:30 a. m.	15	
		8:00 a. m.	16	
Dry-salted, 150 lbs. salt per hogshead. Herring 2½ feet deep in boat	47%	9:30 a. m.	11	At factory
		10:36 a. m.	14	
		11:30 a. m.	18	
		12:30 p. m.	23½	
Dry-salted.....	90%	5:56 a. m.	11	At weir (water same temperature)
		6:24 a. m.	12	
		9:23 a. m.	18½	
		10:36 a. m.	22	
		11:30 a. m.	24½	
		12:30 p. m.	27	
		4:20 p. m.	37½	
				At smoked herring factory

Determinations, which are recorded in Table 1, show that changes of temperature occur in masses of herring in the boats after being removed from the weirs, giving an increase sufficient to produce incubation and allow multiplication of bacteria. *B. walfischrauschbrand* or *Bacillus B.*, if present in feed in the stomachs of the dead herring, probably began to multiply with the production of gas as soon as the temperature reached 25 C. This was undoubtedly the source of the gas bubbles mentioned above in the stomach and intestines. The gas thus produced by the decomposition of the feed may play an important mechanical part in the production of "belly-blown" sardines.

Herring which contain the copepods or shrimp when taken from the weirs decay very rapidly under ordinary conditions of transportation. The digestive tract sometimes becomes distended with gas, the body walls soften and fall away from the ribs as if they were a viscous liquid instead of flesh. Such herring are known as "belly blown." Herring which were full of feed were however, transported for periods of 10 hours and held 12 hours longer in the laboratory when floated in water at 15 C. This method presented a rise of temperature sufficient to permit rapid bacterial multiplication. It also eliminated mechanical injuries to the fish.

"Feedy" fish were taken from the weirs in sterile jars and carried un-iced and unsalted to the laboratory. Samples were then taken aseptically from the stomachs and intestines and from different portions of the body. In all cases

⁶ Arch. f. Hyg., 1910 1911, 73, p. 183.

with a few exceptions, the flesh was free from bacteria. The contents of the digestive tract almost invariably contained *B. walfischrauschbrand* or *Bacillus B.* or both, but in no instance were they isolated from the flesh.

Chemical tests⁷ were made on masses of the feed removed from the stomachs aseptically when the above bacteria were isolated. Both ammonia and amines were found. Large quantities of schizopods and of copepods collected aseptically from the stomachs of the herring were incubated without added mediums or bacterial cultures. Chemical tests were then made from time to time of the decomposing substances. After 48 and 72 hours' incubations (Table 2), both kinds of feed contained large amounts of ammonia and smaller but appreciable quantities of amines. No chemical differences were detected between the decomposition of copepods and schizopods. Bacteriologic examinations made at the time of the removal of the chemical samples from the incubating masses showed *B. walfischrauschbrand* and *Bacillus B.* as the predominating organisms.

TABLE 2

AMMONIA AND AMINES IN SAMPLES OF "FEED" KEPT AT INCUBATOR TEMPERATURE (37.5 C.)

Sample No.	Description	When Taken			Incubated 24 Hours			Incubated 48 Hours			Incubated 72 Hours		
		Milligrams Volatile Nitrogen per 100 gm. as			Milligrams Volatile Nitrogen per 100 gm. as			Milligrams Volatile Nitrogen per 100 gm. as			Milligrams Volatile Nitrogen per 100 gm. as		
		Total	Ammonia	Amines	Total	Ammonia	Amines	Total	Ammonia	Amines	Total	Ammonia	Amines
1	Plankton from Woods Hole, Mass., kept at 30 C. Sample mostly composed of diatoms.....	2.82	4.14		
3	Plankton from St. Croix River off Campobello Island. Mostly composed of copepods.....	6.04	402.2		
7	Copepods from east and south of Campobello Island.....	3.94	272.4	206.2	66.2	468.2	375.1	93.1			
13	Copepods from shallow water east of Grand Manan...	216.6	153.8	62.8						
18	Copepods from north end Grand Manan in very deep water.....	9.50	191.7	143.4	48.3	296.8	260.9	35.9			
5	Schizopods from Wilson's Beach Island, off Campobello.....	1.98	822.9	602.3	220.6
14	Schizopods from Wilson's Beach Island, off Campobello.....	3.95	220.4	88.2	132.2	787.6	573.7	213.9	1009.2	832.6	176.6
21	Feed taken from "belly-blown" fish at wharf, Eastport, Maine.....	37.7	17.1	20.6									
10	Fresh water herring from Taunton River, Taunton, Mass.	9.94	127.0	111.6	15.4	888.3	812.6	75.7

⁷ All chemical analyses were made by Mr. J. B. Wilson, assistant chemist, Animal Physiological Laboratory, Bureau of Chemistry, Washington, D. C.

Pure cultures of these bacteria were inoculated into the sterilized flesh of fish after enrichment with 0.2% dextrose and incubated at 37½ C. After varying intervals of incubation, chemical analyses were made, a bacteriologic check being made at the time of the removal of the chemical samples. The chemical results are recorded in Table 3. They show that both ammonia and amines were formed and that the amines were again less in amount than the ammonia. Positive tests for indol and skatol were also obtained.

TABLE 3
DETERMINATION OF AMMONIA AND AMINES IN PURE CULTURES OF *BACILLUS WALFISCHRAUSCHBRAND* AND *BACILLUS B.* GROWN IN THE LABORATORY

Laboratory No.	Kind of Bacteria	Time of Incubation, Days	Volatile Alkaline Material in Terms of					Nitrogen Determined by the Amino-Acid Method as Nitrogen per 1.0 gm. X ₁ , Mg.	Alkalinity as N/20 Acid per 1.0 gm., C c
			Milligrams Volatile Nitrogen per 100 gm. as			Percentage of Total as			
			Total	Ammonia	Amins	Ammonia	Amins		
30	Sterile	7	18.5	—	—	—	—	0.55	0.0
28	B. walfisch-rauschbrand	2	259.5	215.2	82.9	17.1	17.1	5.34	
28	B. walfisch-rauschbrand	4	—	—	—	—	—	5.65	
31	B. walfisch-rauschbrand	7	510.1	447.1	63.0	87.7	12.3	9.95	2.1
32	B. walfisch-rauschbrand	7	334.6	286.2	18.4	85.6	14.4	5.16	1.5
24	Bacillus B.	2	146.8	—	—	—	—	—	1.2
24	Bacillus B.	4	208.4	160.3	48.1	76.9	23.1	—	1.9
25	Bacillus B.	3	159.1	127.7	31.4	80.3	19.7	—	
29	Bacillus B.	2	145.8	132.6	13.2	81.0	9.0	2.52	
27	X ₂ Bacillus B.	2	225.2	198.8	26.4	88.2	11.8	5.07	
27	Bacillus B.	3	—	—	—	—	--	5.59	

X₁ These determinations made by the later Van Slyke method and apparatus, J. Biol. Chem., 1913, 16: 121.

X₂ A few micrococci in this culture.

In connection with feed in the stomach an interesting observation was made. Two silver hake were caught in herring weirs, one with 21 and the other 23 herring in its stomach. The stomachs were distended until they were approximately 4 inches in diameter at the widest portion. The section of the herrings which lay against the stomach lining had been markedly digested, but the remainder of the fish were perfectly firm and solid. In several of the herring there were copepods, but no gas either there or in their intestines. From two stomachs taken from herring lying in the central portion of one hake's stomach were found *Bacillus B.* and *B. coli*.

Among the references to the occurrence of *B. coli* in fish, Johnson⁸ reports that G. C. Whipple found no *B. coli* in trout, perch and sunfish which were taken from an unpolluted water. Amyot's⁹ conclusions, founded on the examination of 23 fish of 14 varieties caught near Port Stanley, Ontario, on Lake Erie, in August, 1901, agree with Professor Whipple's statement. Johnson, however, found *B. coli* to be present and to multiply in 15 varieties taken from the polluted waters of the Mississippi and Illinois Rivers. All of the fish

⁸ Jour. Infect. Dis., 1904, 1, p. 348.

⁹ Tr. Am. Pub. Health Assn., 1901, 27, p. 400.

which were found in this investigation to contain *B. coli* were taken from in or near sewage polluted water or water enriched with the trimmings of fish.

In an attempt to learn if the sterility of the digestive tract of other fish depends on the presence of feed, cod, rock fish, bass and Taunton (Mass.) River herring or alewives were examined. The alewives alone were obtained without feed present. Of 72 examined, 47 contained neither feed nor bacteria, 3 contained *B. coli*, and 22 contained bacteria but no gas-producing organisms.

From 2 cod, obtained at Eastport, Maine, one of which was freshly caught and one which had been out of water 2 hours, the following results were obtained. Both contained *B. coli* and *B. walfischrauschbrand* in the intestines and *B. walfischrauschbrand* in the gills. Both had partially digested feed in the stomach, of which the portions examined were sterile.

The curing of cod by drying¹⁰ does not always kill the spores of *B. enteritidis sporogenes*. Cod are classed as bottom fish and are known to feed on refuse fish trimmings. Pollock, however, are often cured and sold as dried cod. These fish have often been seen feeding on schizopods in regions where the schizopods were found to contain *B. walfischrauschbrand*. It is therefore suggested advisable to examine commercial "dried cod" for *B. walfischrauschbrand*.

CONCLUSIONS

Swelling of processed cans of sardines is caused by an anaerobic spore-forming organism, which is probably identical with *B. walfischrauschbrand* of Nielsen.

B. walfischrauschbrand was isolated from factory dirt, from the gills of the herring, from feed (schizopods and copepods) found in the stomach and intestines of the fish where it had produced gas, from the thoracic and digestive portions of the schizopods and from the copepods as taken from the water.

Bacillus B. which was also found in the feed, especially in the copepods, pathogenic in peritoneal inoculation (guinea-pig) and produces gas in protein medium containing blood. It is killed at 65 C. in 20 minutes.

The fish while massed together in boats during transportation from the weirs to the cannery heat enough to permit rapid growth of *B. walfischrauschbrand* and *Bacillus B.* with the production of gas in the dorsal ends of the stomachs and intestines. Excess of such development tends to produce "belly-blown" fish, due to the active decomposition of the red-feed first and then of the fish.

The digestive tract of the herring was usually found sterile when no feed was present. The flesh was practically free from bacteria but organisms were present in the gills.

Removal of heads, gills, and viscera from such fish would reduce the danger of spoilage as due to these organisms. This danger is partially reduced by holding the fish in pounds until free from feed.

¹⁰ Stewart, C. Balfour: Thompson-Yates Laboratories, Report No. 3, 1901, p. 32.

B. walfischrauschbrand and *Bacillus B.* both survived frying in oil when the layer of fish was deep enough for 3 fish to overlap. They appear to be killed by heat to 240 F. for 3 minutes, if no fish are allowed to overlap.

B. walfischrauschbrand in the sealed cans will survive $1\frac{1}{2}$ hours in a boiling tank, if a portion of the can is exposed, thus allowing a temperature even 1 or 2 degrees under the boiling point. To obtain efficiency all cans must remain entirely submerged and the water must boil freely.

B. coli and in some cases *B. welchii* were abundant in the water used in some canneries. For reasons of decency, as well as to reduce the loss of canned goods, the water used should be obtained far enough away from sewers to be free from sewage organisms.

DESCRIPTION OF ORGANISM

Bacillus Identified as *B. Walfischrauschbrand*

Morphology: Rod-shaped, gram-positive, occurs singly. Spores always present.

Size: 3.8-5 mikrons long by 0.8 mikrons wide.

Motility: Absent.

Spore Formation: A round spore located near one end of cell giving tennis-racket-shaped organism closely resembling the bacillus of symptomatic anthrax. Size of cell at spore end 1.8 mikrons.

Resistance: Resists heating in steam autoclave 10 minutes, killed by 15 minutes' heating. Culture in dextrose agar resisted 5 lbs. pressure for 5 minutes, killed at end of 8 minutes.

Relation to Temperature: Growth rapid at $37\frac{1}{2}$ C. with production of spores. Slow growth at 25 C.

Reaction to Air: Obligate anaerobe. Grows in agar shake tubes to within 3 cm. of surface.

Color: White.

Agar Containing: 1% of dextrose: Small white pine colonies in depth. Abundant gas at end of 18 hours' incubation at $37\frac{1}{2}$ C. Spores. Odor foul.

Agar Streak: White, moist, slimy round colonies.

Milk: Coagulated.

Nutrient Broth: Slightly turbid, growth at bottom, foul odor.

Lactose Agar: Gas, acid to litmus.

*Bacillus B.**

Morphology: Short, gram-positive rod.

Motility: Absent.

Spores: Not demonstrated.

Resistance: Thermal death point 65 C. for 20 minutes.

* A large portion of this identification was prepared by W. G. Smilie, Harvard Medical School, Boston, Mass.

Cultivation: Grows best at 37½ C. in mediums containing 1 or 2 drops sterile horse blood.

Relation to Air: Obligate anaerobe, growing in agar tubes to within 2 cm. of surface.

Thermal Death Point: 65 C. for 20 minutes.

Blood Plates: 1 c c horse blood to 10 c c meat infusion agar. Forty-eight hours' growth. Small disk-shaped colony, margins regular. Around each colony is a zone of hemolysis. From 1-1.5 mm. in diameter.

Nutrient Agar: Grows only in depth of mediums within 2 cm. of surface. Abundant gas.

Dextrose: Abundant gas, foul odor.

Indol: Negative.

Animal Inoculation: 1 c c of a 24-hour bouillon culture to which 2 drops of horse blood were added was used to inoculate a guinea-pig intraperitoneally. Death in 18 hours. The animal was allowed to lie at room temperature (65 F.) for 18 hours for gas formation.

There were about 5 c c of hemolyzed discolored fluid in the abdomen; abdominal cavity was moderately distended with gas, not in the intestines alone, but free in the peritoneal cavity. Smear from the peritoneum shows large numbers of rods, which take the stain very readily.

Heart blood culture is positive for the gas forming rod.

There was used 0.5 c c of a 24-hour dextrose bouillon plus 2 drops of horse blood to inoculate a mouse intraperitoneally. Death ensued in 48 hours. The abdomen was not markedly distended. The animal was allowed to stay at the room temperature for 18 hours.

The abdomen was markedly distended. Gas is free in the peritoneal cavity and there is also about 0.5 c c of hemolyzed blood, about 1 c c in pleural cavity. Smear from peritoneal and pleural cavity shows large numbers of short bacilli deeply staining.

Heart blood culture is positive for a gas-forming bacillus. Filtered 48-hour bouillon culture through W. Berkefeld filter.

Injected mouse, 0.5 c c, intraperitoneally.

Injected guinea-pig, 1 c c, intraperitoneally.

Mouse died in 12 hours. No fluid in peritoneum.

Necropsy negative.

Guinea-pig was very ill but survived the inoculation.

NOTE.—Since the completion of this work a reference has been found wherein the author states that the intestinal tract of certain fishes is free from bacteria when no food is present. Gillepsie, A. L., Fishing Board of Scotland, Report, 1898, p. 23.

THE EFFECT OF CONCENTRATED SOLUTIONS OF CERTAIN MAGNESIUM SALTS ON PYOGENIC AND OTHER BACTERIA

ZAE NORTHRUP

From the Michigan Agricultural College, East Lansing, Mich.

The feminine world for some time has known of and used a saturated solution of Epsom salt (MgSO_4) as a substitute for talcum or face powder. A small amount of the liquid is taken in the palm of the hand and rubbed gently all over the face until dry, thus leaving a soft "bloom" on the skin.

Those who were inclined to have pimples found that the use of Epsom salt had a salutary effect on the skin, the pimples drying up and disappearing. This knowledge has not been known to the writer alone. A prominent eye, ear, nose and throat specialist stated that he had gained quite a reputation as a skin specialist from suggesting this treatment after having observed its beneficial effects in several instances.

This seemingly specific action led the writer to investigate the influence of magnesium sulphate on the organism commonly found in ordinary pimples, *Staphylococcus aureus*.

The procedure for the phenol coefficient of MgSO_4 was carried out for *Staphylococcus aureus*, and also for *B. typhosus* with the following results:

TABLE 1
STAPHYLOCOCCUS AUREUS

Strength		Time in Minutes					
Per Cent.	Dilution	2.5	5	7.5	10	12.5	15
Phenol	5.0	—	—	—	—	—	—
	2.5	—	—	—	—	—	—
	1.0	+	+	+	+	+	+
	0.5	+	+	+	+	+	+
MgSO_4	50*	+	+	+	+	+	+
	25	+	+	+	+	+	+
	12.5	+	+	+	+	+	+
	5	+	+	+	+	+	+

* Practically saturation. 50 gm. MgSO_4 were made up to 100 c c with distilled water at 25 C.

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TABLE 2
B. TYPHOSUS

Strength		Time in Minutes					
Per Cent.	Dilution	2.5	5	7.5	10	12.5	15
Phenol	5.0	—	—	—	—	—	—
	2.5	—	—	—	—	—	—
	1.25*	—	—	—	—	—	—
	1 1/9*	+	—	—	—	—	—
	1.0	+	+	—	—	—	—
	0.5	+	+	+	+	+	+
MgSO ₄	50	+	+	+	+	+	+
	25	+	+	+	+	+	+
	12.5	+	+	+	+	+	+
	5	+	+	+	+	+	+

* Data for percentages 1.25% and 1 1/9% phenol were obtained from Hyg. Lab. Bul. 82, 1912, U. S. Public Health and Marine Hospital Service.

These results were so unexpected and also so convincing that the experiment of growing *Staphylococcus aureus* in the presence of different percentages of MgSO₄ in broth was next tried.

Two hundred and fifty mm. of MgSO₄ were made up to 500 c.c. with nutrient broth and percentages from 50% ranging by a difference of 5%, down to 5% were prepared, filtered and autoclaved. These tubes were then each inoculated with a loopful from a broth culture of *Staphylococcus aureus* and incubated at 37 C. Table 3 shows the results.

TABLE 3
STAPHYLOCOCCUS AUREUS

Age	Per Cent. of MgSO ₄ in Broth										
	5	10	15	20	25	30	35	40	45	50	
24 hours	+	+	+	+	?	?	—	—	—	—	Macroscopic exam. Microscopic exam.
		Not necessary			+	+	+	+	+	+	
48 hours	+	+	+	+	+	+	+	+	+	+	Macroscopic exam. Growth in broth transfers made from 25-50% MgSO ₄ broth cultures
		Not necessary			+	+	+	+	+	+	

* At the end of 24 hours, from the 25% on up to 50% MgSO₄ cultures, transfers were made into ordinary broth to see if *S. aureus* was still alive. (Results in last horizontal row of the table.) It was noted that 5-25% MgSO₄ broth showed typical heavy turbidity and ring formation while from 30 up to 50% a decided pellicle was formed, with less turbidity. The pellicle was heaviest on 25, 40 and 45% MgSO₄ broth. Beauverie noted a similar phenomenon when growing the cholera organism in broths containing from 0.5-3% NaCl. A thicker and firmer velum was produced in the salt concentrations up to 3%.

The conclusion, then, is that the beneficial effect of a saturated solution of MgSO₄ on the skin is not due to any action on this particular organism.

The same concentrations of MgSO_4 in broth were inoculated with *Streptococcus pyogenes*, *B. typhosus* and *B. coli* — the first organism to see whether streptococcus skin infections might not be held in check by the salt, the second two organisms to ascertain whether the salt has any effect on the growth of organisms found in the intestine.

TABLE 4
EXPERIMENTS WITH DIFFERENT ORGANISMS

Organisms	Per Cent. of MgSO_4 in Broth										
	5	10	15	20	25	30	35	40	45	50	
<i>Streptococcus pyogenes</i> .	—	—	—	—	—	—	—	—	—	—*	MgSO_4 broth Nothing visible macroscopically in plain broth
<i>B. coli</i>	+	+	+	+	+	?	?	?	?	?*	MgSO_4 broth Plain broth
<i>B. typhosus</i>	+	+	+	+	+	?	?	?	?	?*	MgSO_4 broth Plain broth

* Four days after inoculation (1st 2 days at 37 C., last 2 at room temperature). At this time loop inoculations were made from each tube into broth to see if the organism can stand the various concentrations for any length of time. Broth tubes were incubated at 37 C. Results are given for each organism in the second rows above.

Loop inoculations were made from the highest concentration of MgSO_4 broth that shows growth into the remaining concentrations to see whether each may become acclimatized. All of the streptococcus cultures were reinoculated.

TABLE 5
REINOCULATIONS

Organisms	Per Cent. MgSO_4 in Broth										
	5	10	15	20	25	30	35	40	45	50	
<i>Streptococcus pyogenes</i>	All negative										MgSO_4 broth Plain broth
<i>B. coli</i>	*	MgSO_4 broth Plain broth
<i>B. typhosus</i>	*	MgSO_4 broth Plain broth

* Concentrations above this were inoculated with a loopful from this concentration and incubated at 37 C. for 24 hours, reinoculated into plain broth and placed again at 37 C. for another 24 hours. Reinoculation into broth is necessary as the higher concentrations of MgSO_4 broth are too cloudy to determine growth macroscopically.

This seems to indicate that streptococcus skin infections may be inhibited if any concentration of MgSO_4 be used externally.

To determine whether MgSO_4 in a strength above saturation has a plasmolyzing effect on *Staphylococcus aureus*, several clean cover-glasses were split with a red hot wire, flame-sterilized and placed in a

sterile petri dish. Then 50% MgSO_4 broth was heavily inoculated and a loopful placed on each piece of cover-glass which was put at 37 C. to dry. This took from 6-8 hours. At this time each piece was transferred to a tube of broth and incubated at 37 C.

Of the 8 tubes of broth prepared in this way 2 showed heavy growth in 24 hours, and the remaining 6 showed moderate growth as determined by turbidity. This excludes plasmolysis as a factor in the destruction of *Staphylococcus aureus*.

TABLE 6
 $\text{Mg}(\text{NO}_3)_2$

Organisms	Per Cent. of $\text{Mg}(\text{NO}_3)_2$										Hrs.
	5	10	15	20	25	30	35	40	45	50	
First Trial											
<i>Staph. aureus</i>	+	+—	—	—	—	—	—	—	—	—	24
	+	+	+—	—	—	—	—	—	—	—	48
<i>B. coli</i>	+	+—*	+—*	+—*	+—*	+—*	+—*	+—*	+—*	+—*	24
	+	+—	+—	+—	—	—	—	—	—	—	48
<i>Strept. salivarius</i> ...	+—*	+—*	?	—	—	—	—	—	—	—	24
	?	?	—	—	—	—	—	—	—	—	48
<i>B. typhosus</i>	+—*	—	—	—	—	—	—	—	—	—	24
	same	—	—	—	—	—	—	—	—	—	48
Second Trial											
<i>Staph. aureus</i>	+	+	*	—	—	—	—	—	—	—	24
	+	+	+	—	—	—	—	—	—	—	48
<i>B. coli</i>	+	+	*	*	*	*	*	—	—	—	24
	+	+*	+	+	—	—	—	—	—	—	48
<i>B. typhosus</i>	+	*	*	*	*	—	—	—	—	—	24
	—	—	—	—	—	—	—	—	—	—	48
<i>Ps. pyocyanea</i>	+	*	*	*	*	*	*	*	—	—	24
	+*	*	*	*	*	+	+	—	—	—	48

Explanations: In Tables 6, 7 and 8 + = cloudy liquid, growth present; — = clear liquid, no growth present.

* Not cloudy—viscous or flocculent sediment comes up with a swirl on rotating the tube sharply.

The action of MgSO_4 on common pimples can hardly be explained by its physiologic action, as it has only a sedative or local anesthetic influence which is due to its depressing action on the sensory nerve endings.

Morison and Tulloch's¹ work on the treatment of war wounds with magnesium sulphate seems to indicate that this salt has bactericidal properties. They state as follows:

¹ Jour. Roy. Army Med. Corps, 1916, 27, p. 375.

It possesses the desirable property of interfering with the digestive activity of pus. . . . Magnesium sulfate has not so markedly inhibitory action on phagocytosis as one would expect, and therefore, even if it be absorbed to a slight extent, it would not have a deleterious influence on the process. . . . The magnesium ion has a markedly inhibitory action on the growth of streptococci and *B. coli*, and a slightly inhibitory effect on the growth of *B. pyocyaneus*. It has, however, no easily demonstrable influence in the concentrations examined on the growth of staphylococci, nor on the diphtheroids investigated.

Similar experiments were carried out with other soluble magnesium salts $\text{Mg}(\text{NO}_3)_2$, MgCl_2 as well as MgSO_4 , in order to determine whether the Mg ion itself is responsible for the action observed or whether this particular combination of ions is necessary. Tables 6, 7 and 8 show that the latter hypothesis is probably correct:

TABLE 7

 MgCl_2

Organisms	Per Cent. of MgCl_2												
	5 $\frac{1}{3}$	10 $\frac{2}{3}$	16 $\frac{1}{3}$	21 $\frac{1}{3}$	26 $\frac{2}{3}$	32	37 $\frac{1}{3}$	42 $\frac{2}{3}$	48	53 $\frac{1}{3}$	58 $\frac{2}{3}$	66 $\frac{2}{3}$	
<i>Staphylococcus aureus</i>	+	+	+	+	+	+	+	—	—	—	—	—	24 hours 48 hours
<i>B. coli</i>	+	+	—	—	—	—	—	—	—	—	—	—	24 hours 48 hours
<i>B. typhosus</i>	+	—	—	—	—	—	—	—	—	—	—	—	24 hours 48 hours
<i>Ps. pyocyanea</i>	+	—	—	—	—	—	—	—	—	—	—	—	24 hours 48 hours

TABLE 8

 MgSO_4

Organisms	Per Cent. of MgSO_4										
	5	10	15	20	25	30	35	40	45	50	
<i>Staphylococcus aureus</i> ..	+	+	+	+	+	+	+	+	+	+	24 hours 48 hours
<i>B. coli</i>	+	+	+	+	+	+	+	—	—	—	24 hours 48 hours
<i>Ps. pyocyanea</i>	+	+	+	+	+	+	+	+	+	—	24 hours 48 hours
<i>B. typhosus</i>	+	+	+	+	+	—	—	—	—	—	24 hours 48 hours

The results of Morison and Tulloch with regard to *S. pyogenes* were proven in the use of MgSO_4 , but not in case of *B. coli*; MgCl_2 was the only magnesium salt used that had an inhibitory action. In

fact, this latter salt had the most marked inhibitory action on the growth of all organisms used in the experiment. This suggests that a substitution of MgCl_2 for MgSO_4 might have the advantage under certain conditions.

Further study of the specific action of concentrated solutions of MgSO_4 and other magnesium salts on the infected skin or in wounds may present interesting if not valuable information.

THE DEMONSTRATION OF IMMUNE OPSONINS FOR THE PLEOMORPHIC STREPTOCOCCUS IN EXPERIMENTAL POLIOMYELITIS IN MONKEYS

WILLA M. DAVIS

From the Division of Experimental Bacteriology, Mayo Foundation, Rochester, Minn.

Rosenow, Towne and Wheeler,¹ Mathers,² Nuzum and Herzog,³ and Kolmer, Brown and Freese,⁴ have described a micrococcus isolated quite constantly from brain and cord in epidemic poliomyelitis, and Rosenow and Towne⁵ have isolated a similar organism from paralyzed monkeys following the injection of poliomyelitic virus. Mathers and Tunnicliff⁶ found an increase in opsonin apparently specific for this micrococcus in the serum of patients during the attack of poliomyelitis, and Mathers and Howell⁷ found a specific increase in opsonin in the serum of rabbits immunized with different strains of the pleomorphic streptococcus. Kolmer and Freese⁸ using polyvalent antigens of this streptococcus obtained complement fixation with the serum of a small percentage of persons with poliomyelitis. Solis-Cohen and Heist⁹ found "that the serums of a large percentage of patients with poliomyelitis give high opsonic indexes with this streptococcus but not with streptococci from nonpoliomyelitic sources nor with staphylococci, diphtheroids and gram-negative bacilli obtained from poliomyelitic material."

In an extensive study of the question of antibody production in poliomyelitis, Rosenow and Gray¹⁰ found an increase in the specific agglutinating power toward this organism in the serum of patients with poliomyelitis, in the serum of monkeys with poliomyelitis following inoculation of virus in the usual way, and in the serum of monkeys injected with the "poliococcus." The present study concerns the development of opsonins in the serum of monkeys following the inoculation of poliomyelitis virus.

¹ Jour. Am. Med. Assn., 1916, 67, p. 1202.

² Jour. Am. Med. Assn., 1916, 67, p. 1019. Jour. Infect. Dis., 1917, 20, p. 113.

³ Jour. Am. Med. Assn., p. 1205.

⁴ Jour. Exper. Med., 1917, 25, p. 789.

⁵ Jour. Med. Research, 1917, 36, p. 175.

⁶ Jour. Am. Med. Assn., 1916, 67, p. 1935.

⁷ Jour. Infect. Dis., 1917, 21, p. 292.

⁸ Jour. Immunol., 1917, 2, p. 327.

⁹ Jour. Infect. Dis., 1918, 22, p. 175.

¹⁰ Ibid., p. 345.

Four monkeys were used in the experiment.

Monkey 147, a normal control, was not injected.

Monkey 148, April 21, 1917, was given 0.5 cc of a 5% suspension in salt solution of glycerolated virus intracerebrally. April 28 the animal showed paralysis; it died April 29. The lesions were characteristic and the pleomorphic streptococcus was isolated from brain and cord.

Monkey 149, April 21, 1917, was given 5 cc of sensitized vaccine intravenously; the dose contained streptococci from 75 cc of dextrose broth culture; the organisms had been suspended in immune horse serum for 2 hours at 37 C. and left in the icebox over night; they were then washed in water and suspended in salt solution.

Monkey 150, April 21, 1917, was given 20 cc of normal horse serum intravenously to test whether horse serum alone would produce any increase in opsonin. May 2, four days after the death of Monkey 148, Monkey 150 was given 0.5 cc of a 5% saline solution suspension of glycerolated virus intracerebrally, and in addition an intravenous injection of 12 cc of immune horse serum. The intravenous injection of 12 cc of immune horse serum was repeated May 3, 5, 6, and 7. May 8, the animal showed definite flaccid paralysis, and died May 14. The lesions were characteristic; the results of the cultures were similar to those from Monkey 148.

Blood was collected from these 4 monkeys April 18 and every 2nd day thereafter until April 29 when the paralyzed monkey died. The blood was allowed to clot, placed in the icechest for 24 hours and after the serum had been decanted it was stored in the icebox until June when the counts were made.

TECHNIC

The strains of pleomorphic streptococci used were shown to have retained their specific agglutinating property. They came from cases of epidemic poliomyelitis occurring in New York and Philadelphia, and from monkeys paralyzed by the injection of virus. Eight of the human strains (714, 721, 722, 729, 839, 841, 842, 899) were recovered from the brain and cord and 3 (730, 732, 748) from the tonsils. The 3 monkey strains (M49, M106, M148) were recovered from the brain and cord of monkeys paralyzed with virus or filtrates of virus. The exponents to the right and above the figures designating the strain in Tables 1 and 2 indicate the number of animal passages; the figure following the period designates the culture generation. Three control strains were used, a streptococcus viridans from the tonsil in a case of arthritis, a pneumococcus (622), and a feebly hemolytic streptococcus (257).

Most of these strains had been kept in the laboratory in deep stabs of ascites fluid, plain tissue agar and were transferred to broth 24 hours before the counts were made. Any broth cultures in which the growth was not uniformly diffuse were discarded.

The test tube method was used in making the opsonin determinations. The tubes, containing 0.05 cc each of leukocyte suspension,

serum, and culture, were incubated at 37 C. for 15 minutes, after which the smears were made immediately. The organisms in 50 leukocytes were counted; the figures in the tables, therefore, represent the actual number of organisms taken up by 50 leukocytes. Any cell which contained more than 30 organisms was not included in the count. Almost without exception these crowded leukocytes were found in mixtures containing immune serum.

The bacterial counts made are tabulated in series, 2 series in Table 1, and 3 in Table 2. As the same 24 hour culture, and the same leukocyte suspension were employed throughout, the conditions for each single series were uniform.

TABLE 1
OPSONIC POWER OF THE SERUM OF 4 MONKEYS

Strain	Monkey 147 Normal Control		Monkey 148 Injected with Virus		Monkey 149 Injected with Sensitized Vaccine		Monkey 150 Injected with Normal Horse Serum	
			Before Injec- tion	Eight Days after Injection; One Day after Onset of Paralysis	Before Injec- tion	After Injec- tion	Before Injec- tion	After Injec- tion
	April 18	April 29	April 18	April 29	April 18	April 29	April 18	April 29
899	51	50	39	257	32	65	61	56
M 106.5	38	2	14	120	0	68	0	4
722 ² .4	56	20	20	91	22	119	66	54
730.11	87	59	39	139	19	78	55	54
913 control	17	..	19	12	47	51	10	10
714.3	2	43	9	57	17	6
714 ³ .2	22	64	20	91	64	32
714 ⁴ .2	0	20	10	35	4	4
748 ² .4	30	64	18	30	14	12
622 control	34	38	32	24	10	11

In Table 1 is given the opsonic power of the serum of 4 monkeys obtained in 2 series of experiments. The serum of the normal control, Monkey 147, and the one injected with normal horse serum, Monkey 150, in no case shows any increased opsonic power. On the other hand, the opsonic power of the serum of Monkey 148 injected with virus, and Monkey 149 injected with sensitized vaccine, shows a well marked increase in phagocytic power 8 days after the injection against all of 7 human strains of the pleomorphic streptococcus (3 before and 4 after from 1-4 animal passages) and one monkey strain, but no increase against the control strains.

In Table 2 are given the results obtained in 3 additional series of experiments. In this series, as in those shown in Table 1, the opsonic power of the serum of the normal control (Monkey 147) and Monkey 150 varied only slightly. The serum of Monkey 148 again shows a marked increased opsonic power after injection, and after paralysis occurred. The increase is slight up to the 6th day after the injection of virus, but decided between the 6th and 8th days. The serum of Monkey 149 shows a consistent increase in opsonic power with all of 9 strains 14 days after the injection of sensitized vaccine. It is of interest to note that this monkey showed a degree of immunity to virus in that the incubation period was prolonged for 1 week and the animal recovered, while the control died promptly after the onset of paralysis.

The serum of Monkey 150 showed no change in opsonic power in numerous tests until 12 days after an intracerebral injection of virus and after repeated injections of immune horse serum. At this time there was a marked rise toward all of 5 strains (Table 2), and coincident with this marked increase in opsonin, the animal showed a degree of immunity to virus, since it lived for 6 days after the onset of paralysis, while the control died of a rapidly progressing paralysis in 24 hours. It has previously been observed that immune horse serum does not always protect completely against the forced experiment of intracerebral inoculation of highly virulent virus. The lack of complete protection in Monkey 150 may, however, be due to the fact that the immune horse serum may have been toxic since the animal received an injection of normal horse serum 12 days previously (Tables 1 and 2).

CONCLUSIONS

There occurs a well marked specific increase in opsonin for the pleomorphic streptococcus in the serum of monkeys during attacks of poliomyelitis following the inoculation of virus. Since this increase in opsonin occurs toward strains derived from human cases as well as from experimental poliomyelitis following the injection of virus, the pleomorphic streptococcus in this disease cannot be regarded as an accidental invader of the nervous system.

ON THE BACTERIA IN THE SPUTUM IN MEASLES

RUTH TUNNICLIFF

(Chicago) Contract Surgeon, U. S. Army

In a previous article I¹ have described a small gram-positive diplococcus, which was isolated from the blood, eye, ear, nose and throat of measles patients. The organism was cultivated only in anaerobic cultures from the blood, but generally grew aerobically in the second generation. The diplococci could be cultivated readily from the throat on aerobic blood agar plates — probably due to the presence of other organisms. Diplococci corresponding morphologically to those in cultures could be demonstrated in smears made from the tonsils and anterior pillars.

Rabbits² immunized with 4 measles strains (blood, eye, ear and throat) showed a high opsonic power not only for strains used in immunization, but for other strains of the same coccus isolated from measles blood, nose and throat.³ This reaction was found to be specific — no such phagocytic power being observed for diplococci isolated from various other sources.

An opportunity to study the sputum of measles patients was afforded at Camp Meade, Md. Seventeen specimens were examined on the first or second day after the appearance of the eruption. The sputum was collected in sterile petri dishes, the patient first carefully gargling with sterile normal salt solution. The sputum was washed 5 times in salt solution and then smeared over the surface of human blood-agar plates. Direct smears of the washed sputum showed the presence of a variable number of small (0.5 mikrons) round or very slightly elongated gram-positive diplococci, often in large numbers and frequently the only organisms present. The picture was similar to that of a measles throat smear.

The diplococcus was isolated from all of the sputum examined. *Bacillus influenzae* and *Streptococcus hemolyticus* were each isolated twice.

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¹ Jour. Am. Med. Assn., 1917, 68, p. 1028.

² Ibid., 1918, 71, p. 104.

³ Tunncliffe and Brown: Jour. Infect. Dis., 1918, 23, p. 572.

The colonies of the diplococcus appear in 24 or 48 hours on human blood-agar plates as small, dry greenish colonies surrounded by a green discoloration of the medium, sometimes becoming slightly hemolytic in 48 hours. They become more moist on cultivation. They grow slightly on plain agar as a delicate, colorless growth. The growth is flocculent in both dextrose and plain broth, the fluid generally remaining clear. They ferment lactose, dextrose and saccharose. One ferments salicin. None ferment inulin and mannite. None are soluble in bile.

The growth on 3 blood-agar slants injected intraperitoneally was not virulent for guinea-pigs. The same dose injected intravenously made rabbits ill, but did not kill. One-tenth of the growth of a blood-agar slant injected intraperitoneally, killed mice in 24 hours, producing a peritonitis and septicemia. The diplococcus was recovered from the blood and peritoneal fluid.

TABLE 1
PHAGOCYTOSIS EXPERIMENTS

Strains of Diplococci	Percent of Leukocytes Engaged in Phagocytosis at a Dilution of 1:15	
	Serum of Immunized Rabbit	Serum of Control Rabbit
Immunizing Strains		
Blood	23	7
Ear	21	10
Throat	12	3
Eye	Spontaneous phagocytosis	Spontaneous phagocytosis
Sputum Strains		
1	17	8
2	4	1
3	9	3
4	21	6
5	12	6
6	27	11
7	9	0

Rabbits were immunized by intravenous injection first of killed and then of living diplococci isolated from measles blood, eye, ear and throat. After 4 injections of increasing doses, the rabbits were examined for their opsonic power for the 4 immunizing strains as well as for 7 similar diplococcus strains isolated from the sputum of the measles patients. The serums were diluted 5 times with normal salt solution and mixed with equal parts of washed human leukocytes and bacterial suspensions and incubated 15 minutes. The mixtures were smeared on glass slides, stained with Wright's stain and 50

polymorphonuclear leukocytes counted and the number taking part in phagocytosis noted.

The immune rabbits showed an increased opsonic power from 2-9 times greater than that of the control rabbit, both for the immunizing diplococci and the strains isolated from measles sputum. This is of interest, as no sputum strains were used in immunizing the rabbits. These results correspond to the results with diplococci isolated from blood, throat and nose of measles patients, the immunized measles rabbits showing a specific increased opsonic power for these diplococci. This immunologic test appears to be the best method so far devised to differentiate the measles diplococcus from other similar green producing cocci.

The result shows that small gram-positive diplococci are the predominating organisms in smears and cultures from measles sputum. They correspond morphologically, culturally and immunologically to the diplococci isolated from the blood, throat, eye, ear and nose of measles patients.

THE PERFUSION EXPERIMENT IN THE STUDY OF CELLULAR ANAPHYLAXIS *

WITH ONE PLATE

W. P. LARSON AND E. T. BELL

*From the Departments of Bacteriology and Pathology, University of Minnesota,
Minneapolis*

Although a large amount of work has been done in recent years in attempts to determine the fundamental nature of anaphylaxis, this problem has not been entirely cleared up. It is not the purpose of this paper to attempt in any way to review the vast literature which has accumulated on the subject. In the interest of context, however, it may be recalled that there are two theories regarding the fundamental mechanism of anaphylaxis. Friedberger and his pupils hold to the humoral theory. According to this conception, when an antigen is acted on by its specific antibody of the third order the antigen is broken up into toxic substances which are called anaphylatoxin or apotoxin, to which all manifestations of protein anaphylaxis are ascribed. This conception of the mechanism of anaphylaxis would seem to be supported by the test tube experiment, in which apotoxin may be readily formed by bringing together the suitable reagents. The humoral theory is, however, wholly inadequate to account for the observation that, if an animal is immunized passively, a certain incubation period must elapse before anaphylactic phenomena manifest themselves on the introduction of the antigen.

It was a study of the mechanism of passive anaphylaxis that led to the development of the cellular theory, the chief proponents of which are Schultz,¹ Dale,² Weil,³ and Cocoa.⁴ According to the cellular theory the site of reaction in anaphylactic phenomena is not in the tissue fluids but within the cells. When an animal is immunized passively it is assumed that the tissue cells appropriate the antibodies and thus become sensitized to the antigen. According to the view of the proponents of the cellular theory of anaphylaxis, we have here

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¹ Jour. Pharm. and Exper. Ther., 1910, 1, p. 549; Hygienic Lab., Bull. No. 80, 1912.

² Jour. Pharm. and Exper. Ther., 1913, 4, p. 167.

³ Jour. Med. Research, 1913, 22, p. 497; 1914, 25, p. 87 and p. 299.

⁴ Ztschr. f. Immunitätsforsch. u. exper. Therap., O., 1914, 20, p. 622.

a plausible explanation of the period of latency required to effect passive sensitization, which the Friedberger theory does not supply. If we accept this explanation of anaphylaxis it is apparent that the tissue cells discriminate between the different orders of antibodies, as passive immunization does not always sensitize against the specific antigen. Were this the case it would be the height of folly to administer tetanus antitoxin prophylactically, as such treatment would sensitize the individual to tetanus rather than afford protection. It is, on the contrary, admittedly good therapeutics to immunize passively against tetanus and in some cases against diphtheria as well. The individual immunized against tetanus never becomes more sensitive, but invariably more immune to tetanus toxin. If the tissue cells should appropriate the injected tetanus antitoxin and the individual later become infected with tetanus, the passive immunization would probably prove fatal. It would therefore appear that antibodies of the first order are not involved in the mechanism of cellular anaphylaxis. The question as to the reason for this discrimination by the tissue cells between the antibodies of the first and third order would indeed be an interesting problem for investigation.

While there are some very potent objections to the unreserved acceptance of the cellular theory of anaphylaxis, it has, on the other hand, much in its favor. The fact that a period of incubation of at least from 4-6 hours is required to sensitize passively would seem to allow of no other explanation. This is further supported by the observation of Weil that tissue sensitization is coincident with the disappearance of antibodies from the blood stream. Weil found further that a sensitized animal could be protected from anaphylaxis by insuring the presence of antibodies in the blood stream when the antigen was given.

The final supporting pillar to the theory of cellular anaphylaxis was furnished by the work of Schultz and Dale who attempted to wash the sensitized tissues free of blood and lymph, after which such tissues were exposed to the stimulus of their specific antigens. These experiments presuppose the ability completely to isolate the tissue cells from the body fluids. Dale washed the uterus of the guinea-pig supposedly free of blood by inserting a cannula into the abdominal aorta and perfusing the lower half of the body with Locke's fluid, allowing the efferent fluid to escape from the inferior vena cava. The perfusion was continued "for upwards of half an hour" until the efferent fluid appeared free of blood. The same technic has been used by Weil and others in the study of cellular anaphylaxis in guinea-pigs.

No attempt was made by these authors to estimate the amount of residual blood remaining in the tissues following perfusion. Such investigation was undertaken by Cocoa. By estimating the amount of blood cells and complement in the perfusion fluid he arrived at the conclusion that from 1.6-6% of residual blood remained in the tissues following perfusion.

Manwaring⁵ perfused the lungs of normal and anaphylactic guinea-pigs by putting the afferent cannula into the pulmonary artery and allowing the fluid to escape through an incision in the left auricle. Assuming that he had washed the lungs free of blood with Locke's solution, he concluded that the anaphylactic reaction was partly cellular and partly humoral.

In studying spontaneous phagocytosis in the liver with India ink we were surprised to find that the ink particles were deposited only in restricted areas of the organ. It was evident that only a small part of the liver was traversed by the perfusing fluid. This observation suggested the possibility of making use of this procedure in studying the course of the perfusing fluid through an organ and in determining to what extent it is possible to wash an organ free of blood.

EXPERIMENTS

The apparatus is so constructed that fluid, at body temperature, may be passed through the organ and collected for examination. The animal is placed inside an incubator kept at a temperature of 37 C. A funnel provided with a stopcock is supported on a ring-stand. Fluid from the funnel passes through a glass coil which rests in a beaker of water kept at a temperature of 38 C. A rubber tube leads from the glass coil through a small hole in the side of the incubator to the afferent cannula. This tube is connected with a mercury manometer and the height of the funnel is so adjusted that the manometer registers a pressure of about 2-3 cm. Hg. The efferent cannula is connected with a rubber tube which passes through a small hole in the side of the incubator so that the fluid may be collected outside. Several places are provided to let out any air bubbles which may get into the apparatus. By means of the stopcock on the funnel the perfusion may be discontinued temporarily at any time. Locke's solution, at a temperature of about 37 C., was used to perfuse the organs. Most of the experiments were performed on the liver, but the lungs and the uterus were also perfused a few times each. A typical experiment will illustrate the procedure and the results.

The rabbit is killed with ether. Before the heart quits beating a cannula containing sodium citrate is inserted into the portal vein near its entrance into the transverse fissure of the liver. The gastric vessels in the lesser omentum are then tied off. Another cannula is inserted into the superior vena cava in the thorax. From 50-100 cc of Locke's solution warmed to 37 C. are passed through immediately under gentle pressure to wash out most of the blood. The animal is then put into an incubator (temperature 37-40 C.),

⁵ Jour. Immunol., 1917, 2, p. 157.

and the perfusion is begun at once, about 10 minutes after the insertion of the first cannula. The manometer registers a pressure of about 2-3 cm. Hg. Perfusion begins at 2:55. Fluid from exit cannula is reddish at first but finally becomes clear. At 3:07 a faint trace of albumin in fluid from exit cannula; 3:15, albumin negative. Perfusion stopped for 3 minutes, then continued. Fluid is now blood stained, shows erythrocytes microscopically, and gives positive albumin test. 3:22, albumin negative; perfusion stopped for 5 minutes. 3:27, perfusion begun again. Fluid contains blood. Albumin strongly positive. 3:35, albumin negative. Perfusion discontinued for 6 minutes. 3:41, perfusion continued. Blood and albumin present. 3:48, albumin negative. Perfusion discontinued for 5 minutes. At 3:53, perfusion continued. Blood and albumin present. 4:00, albumin negative. Perfusion discontinued for 5 minutes. 4:05, perfusion continued. Blood and albumin present. 4:06, end of experiment. About 2,000 cc of Locke's solution has been passed through the liver.

All perfusions, whether of liver, lung, or uterus, give about the same results. It is never possible to wash out all the blood and albumin. The fluid from the exit cannula soon becomes free of albumin and blood, but if the perfusion is discontinued for a few minutes both these substances reappear. The explanation of this phenomenon will be more apparent after we have considered the experiments with India ink.

TYPICAL INDIA INK EXPERIMENT

A liver is prepared as described above and washed with 1,000 cc of Locke's solution. The fluid from the exit cannula is free from albumin. Then a little India ink (Higgins) is added to another 1,000 cc of Locke's solution and this is passed through the liver. The ink suspension is very thin. At first the fluid from the exit cannula is clear, all the ink particles having been taken up by the endothelial cells of the liver. Later on the fluid from the exit cannula is just as dark as that entering the afferent cannula, showing that no more ink particles are being held in the liver. The perfusion is discontinued at this point. The pressure is constant throughout the experiment and the rate of flow from the exit cannula is unchanged, indicating that there is no obstruction to the flow of the fluid.

A liver perfused with Locke's solution containing India ink shows blackened portions, the black color being due to retention of the carbon particles of the ink by the endothelial cells of the liver. The blackened areas evidently correspond to the parts of the liver through which the perfusing fluid passed. There is great variation in the size and position of the blackened areas in different specimens. In every case there are large areas of liver tissue not blackened at all. In some instances only the dorsal part of the liver is blackened (this is the lower part during the perfusion); in others only a comparatively small area around the hilus. The blackened areas shown in Figs. 1, 2, 3, and 4 indicate the parts of the liver through which the perfusing fluid actually passed. It is clear that even in the most favorable

cases there are extensive areas of liver tissue not reached by the perfusing fluid and that sometimes only an insignificantly small part of the liver is perfused. Even in blackened portions of the liver the cut surface shows that the fluid has reached only the central parts of the lobule (Fig. 5). It is therefore an error to assume that all the blood has been washed out of the liver because the fluid coming from the organ is free from albumin.

It was pointed out in the foregoing that it is not possible to wash all the blood out of the liver. It is easy to get the fluid from the liver free from albumin by continuous rather rapid perfusion; but if the perfusion is discontinued for a few minutes blood and albumin invariably reappear. The experiments with ink give us a clear explanation of this phenomenon. The perfusing fluid traverses only certain parts of the liver and these soon become free of blood; but during the periods when the perfusion is discontinued, blood from the adjacent areas of liver tissue diffuses into the main path of the fluid. Hence, at each renewal of the perfusion after a pause blood and albumin reappear.

On theoretical grounds it is not to be expected that all of the blood can be washed out of an organ with Locke's solution since the latter is much thinner than blood and produces much less friction on the walls of the vessels. As soon as a passage is opened up through the organ the resistance along this route will be much less than through capillaries containing blood, and the fluid will take the path of least resistance.

Air bubbles cannot be a factor in directing the course of the perfusing fluid since these were always carefully excluded. The ink does not block any of the blood vessels. The carbon particles are found on microscopic examination for the most part in the endothelial cells. A liver blackened by an ink perfusion is unaffected by washing with clear Locke's solution. No carbon particles are washed out.

The lungs were perfused by putting the afferent cannula in the pulmonary artery and the efferent in the left auricle. The trachea was ligatured before the thorax was opened to prevent collapse of the lungs. It was also found impossible to wash all the blood out of the lungs. The efferent fluid soon became free of blood and albumin, but both these substances reappeared after temporary interruption of the perfusion. Perfusion with India ink, after preliminary washing with Locke's solution, showed the same irregular blackening of the lung tissue as was described above in the liver (Fig. 6).

The uterus was perfused according to Dale's method. In general, the same results were obtained as with the lungs and the liver.

Since the perfusion of an organ is so incomplete it is obvious that circulating antibodies are not entirely removed by this procedure. It follows that the experiments of Dale and Weil with perfused uterine muscle cannot be regarded as lending any support to the theory of cellular anaphylaxis. In like manner it will be seen that the presence of cellular antibody is not established in Manwaring's experiment with 14-day anaphylactic lung.

It is recognized by us that our experiments do not overthrow the doctrine of cellular anaphylaxis. It is our purpose only to call attention to the unsoundness of one of the main arguments which have been advanced in its support.

SUMMARY

It has been shown that it is impossible to wash all the blood out of an organ by perfusion methods. The efferent fluid becomes free from blood and albumin in a short time, but these substances reappear if the perfusion is temporarily discontinued a few minutes.

By perfusing organs with Locke's solution containing India ink, the course taken by the fluid through the organ has been mapped out. It has been found that a surprisingly small part of the capillary system is really washed out by the perfusing fluid.

This technic does not therefore remove circulating antibodies completely, as has been assumed, and this type of experiment does not establish the presence of cellular antibodies.

EXPLANATION OF PLATE

The organs were first perfused continuously with warm Locke's solution until the efferent fluid was free from albumin, after which they were perfused with warm Locke's solution to which some India ink had been added. The blackened areas show the course of the fluid through the organ. All figures are photographs.

Fig. 1. Ventral surface of liver.

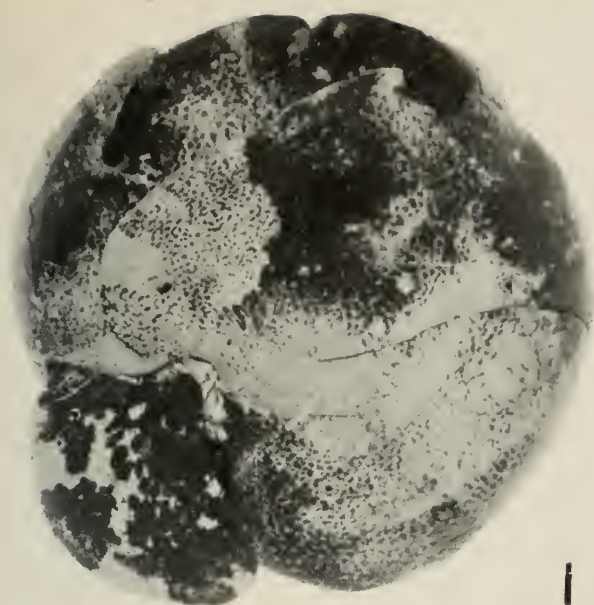
Fig. 2. Dorsal surface of liver. Very little passed through the left lobe.

Fig. 3. Two pieces of a liver, showing a very incomplete perfusion.

Fig. 4. Dorsal surface of liver. The fluid passed through a relatively small area around the transverse fissure.

Fig. 5. Section of a deeply blackened area from a liver, showing that even in this part not over half the hepatic cells are reached by the perfusing fluid.

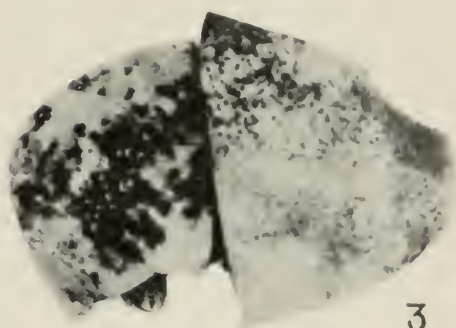
Fig. 6.—Dorsal surface of lungs. Very incomplete perfusion of one lung.



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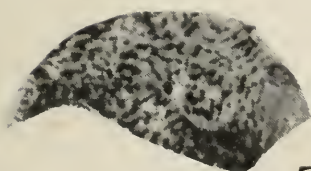
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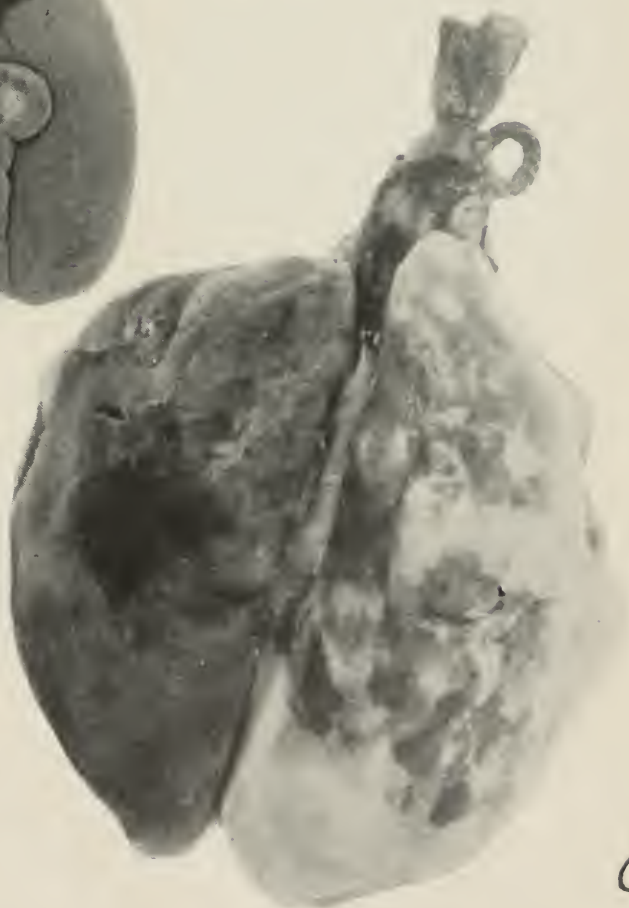
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6

CHLORINATED LIME AND HALAZONE IN THE DISINFECTION OF DRINKING WATER

BERNARD FANTUS

From the John McCormick Institute for Infectious Diseases, Chicago

The desirability of possessing, in a convenient and readily portable form, a reliable disinfectant for small quantities of water, such as might be carried in a soldier's drinking bottle, has led Dakin and Dunham¹ to advocate p-sulphondichloraminobenzoic acid ($\text{Cl}_2\text{N} \cdot \text{O}_2\text{S} \cdot \text{C}_6\text{H}_4 \cdot \text{COOH}$), under the name of halazone, as the best agent they were able to find, suitable for this purpose.

The present work was undertaken chiefly to compare the value of this new agent with that of chlorinated lime. To arrive at a practical conclusion, the following questions had to be answered regarding these two bodies: What is their relative germicidal power? What is their comparative toxicity? How do they compare in regard to suitability for tablet-making (for a tablet is the ideal form for the purpose in mind)?

COMPARATIVE GERMICIDAL POWER

To ascertain this, I at first resorted to the determination of the phenol coefficient, according to the Hygienic Laboratory method.² A considerable amount of time devoted to this attempt, made it evident that the method was unsuitable for the purpose, on account of the following reasons: (1) This method arbitrarily fixes short time limits for action, $2\frac{1}{2}$ and 15 minutes; when, in point of fact, the bodies studied developed their best action after the specified period. (2) It presupposes that there exists a simple and parallel relation of action according to concentration of solution and time of action. This, it has been found, does not exist in case of the two substances under consideration. (3) The medium, in which the disinfectant is permitted to act, is far too heavily polluted with bacteria (one-tenth ml. of 24-hour typhoid culture to 5 ml. of liquid), to make the determination of practical value for purposes of water disinfection. (4) The disin-

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¹ Brit. Med. Jour., 1916, 1, p. 160; 1917, 1, p. 682, and 1917, 2, p. 790; also Am. Jour. Med. Sc., 1917, 154, p. 181.

² Anderson, J. F., and McClintic, T. B.: Hygienic Laboratory Bulletin, No. 82, Apr., 1912.

fectant is added to the medium before the bacteria are introduced. This causes an unfair destruction, by materials present in the medium, of such unstable chemicals as the chlorin bodies studied. In practical work the bacteria are present before the disinfectant is added.

The standard method of examining disinfectants proposed by the committee of the American Public Health Association³ suffers from similar defects for the purpose in view. It must, of course, be recognized that this method is intended merely to serve for the preliminary classification of disinfectants; and that, for special purposes, special methods have to be devised.

To meet the requirements of the purpose in mind, the following modifications of the standard method were elaborated: No time limit was set to the experiment. On the contrary, the time was extended, until it became evident that further increase in time was useless, thus employing a natural rather than an arbitrary time limit. The results were charted on a logarithmic basis according to time and concentration, which permits showing at a glance the relationship of bodies differing so enormously in degree of action, as do these substances in comparison with phenol. A fairly large amount of water, 100 ml., was infected with 0.1 ml. of 24-hour broth culture of typhoid organisms, which gave a water pollution of about 20,000-30,000 per ml. The disinfectant was added after the organisms had been introduced.

Technic.—A 24-hour typhoid culture grown in standard extract broth, of a reaction of + 1.5 acid to phenolphthalein, was filtered through sterilized filter paper; and 0.1 ml. accurately measured by means of a Fournier syringe, discharged into 100 ml. of water contained in wide-mouthed sterile glass-stoppered bottles. These were brought to a temperature of 20 C., and kept at about this temperature by means of a water-bath like that described by Anderson and McClintic.² It was, however, difficult to maintain this temperature absolutely at this point in the long-time experiments during hot weather. At times, the temperature rose to 21 or 22 C. This may account for some of the variations in results. After a control culture had been taken, the accurately weighed or measured disinfectant was added; the contents of the bottle mixed by shaking, and subcultures taken at intervals, shorter in the higher concentrations and longer in the greater dilutions. The results were read after 48 hours' incubation by noting the turbidity of the tubes in which growth had taken place. Growth was recorded by + or ×; no growth by O. When tap water was used, growth was recorded by T; no growth by □.

Inasmuch as chlorin is essentially the active agent in halazone as well as in chlorinated lime, it is of considerable theoretic as well as practical interest to determine whether the chlorin in the form of chloramin is as active as in that of the hypochlorite. Hence strictly equiatomic quantities were compared with each other. Thus an N/10 halazone powder was prepared in the following manner. A little more than 0.0675 gm. each of halazone and of anhydrous sodium carbonate were mixed with 9.865 gm. of sodium chlorid. This was titrated with

³ Am. Jour. Publ. Health, 1918, 8, p. 506.

N/10 sodium thiosulphate and enough sodium chlorid added to make 1 gm. of the powder exactly equivalent to 1 ml. of the solution. In a similar manner, an N/10 powder of chlorinated lime was prepared by so adjusting the "available chlorin" in the mixture with sodium chlorid that each gm. would correspond to 1 ml. of N/10 sodium thiosulphate, when tested in the usual manner, by means of sodium iodid and acetic acid, using starch solution as an indicator. Each time before conducting a test, the powder was reassayed; and, whenever deterioration had occurred, allowance was made for it.

In plotting the results on cross-section paper, it became necessary to use a logarithmic scale, in order to bring the chart within bounds of convenience. In Charts 1 and 2, the concentration of the disinfectant has been indicated as ordinates on a logarithmic scale; and the time as the abscissas, likewise logarithmically. The figures at the left of the chart indicate fractions of N; the higher the figure the lower the concentration. The figures at the bottom of the charts indicate minutes. Only the crucial points of the various observations are noted; that is, a large number of + signs might have been placed to the left side of the one recorded, and a large number of zeros to the right of the O shown in the table, if all the tests made had been noted on the chart. For the sake of clearness, this has not been done. Where two or more experiments did not quite correspond, this is indicated. A few great aberrations from the average results were discarded, as in some of these error of technic was discovered; in others, such error was probable even though not discovered. The line or curve expressing the action of the agent was drawn as nearly as possible midway between the + and O signs, but so as to leave few or no + signs to the right of the line. The position on the table of a line thus drawn, or rather a zone to each side of it, may be taken to represent the activity of the drug. It is approximately correct not only for the concentration tested, but also for intermediate time and concentrations, as has become evident over and over again. The relative position of these lines to each other may be taken to represent the relative activity of the substances tested.

The following signs are used on Charts 1 and 2 to distinguish graphically between the various data to be shown:

- + = Growth—disinfectant acting in distilled water.
- O = No growth—disinfectant acting in distilled water.
- ⊕ = Growth and no growth in two different experiments.
- T = Growth in tap water.
- = No growth in tap water.
- × = Growth after potassium permanganate in distilled water.
- ◇ = No growth after potassium permanganate in distilled water.
- = Line of action in distilled water.
- = Line of action in tap water.

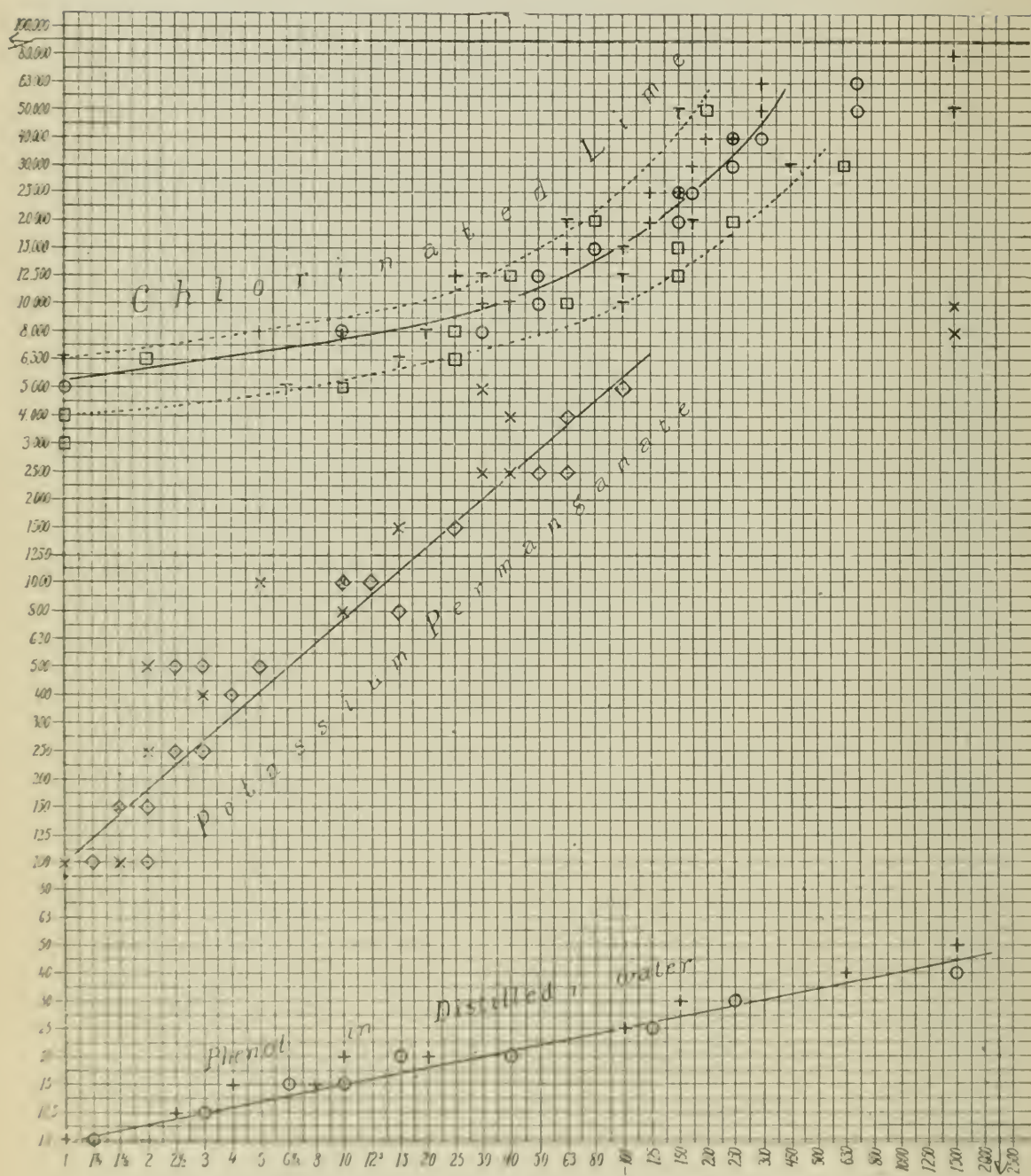


Chart 1.—Record of the experiments made with phenol, chlorinated lime, and permanganate.

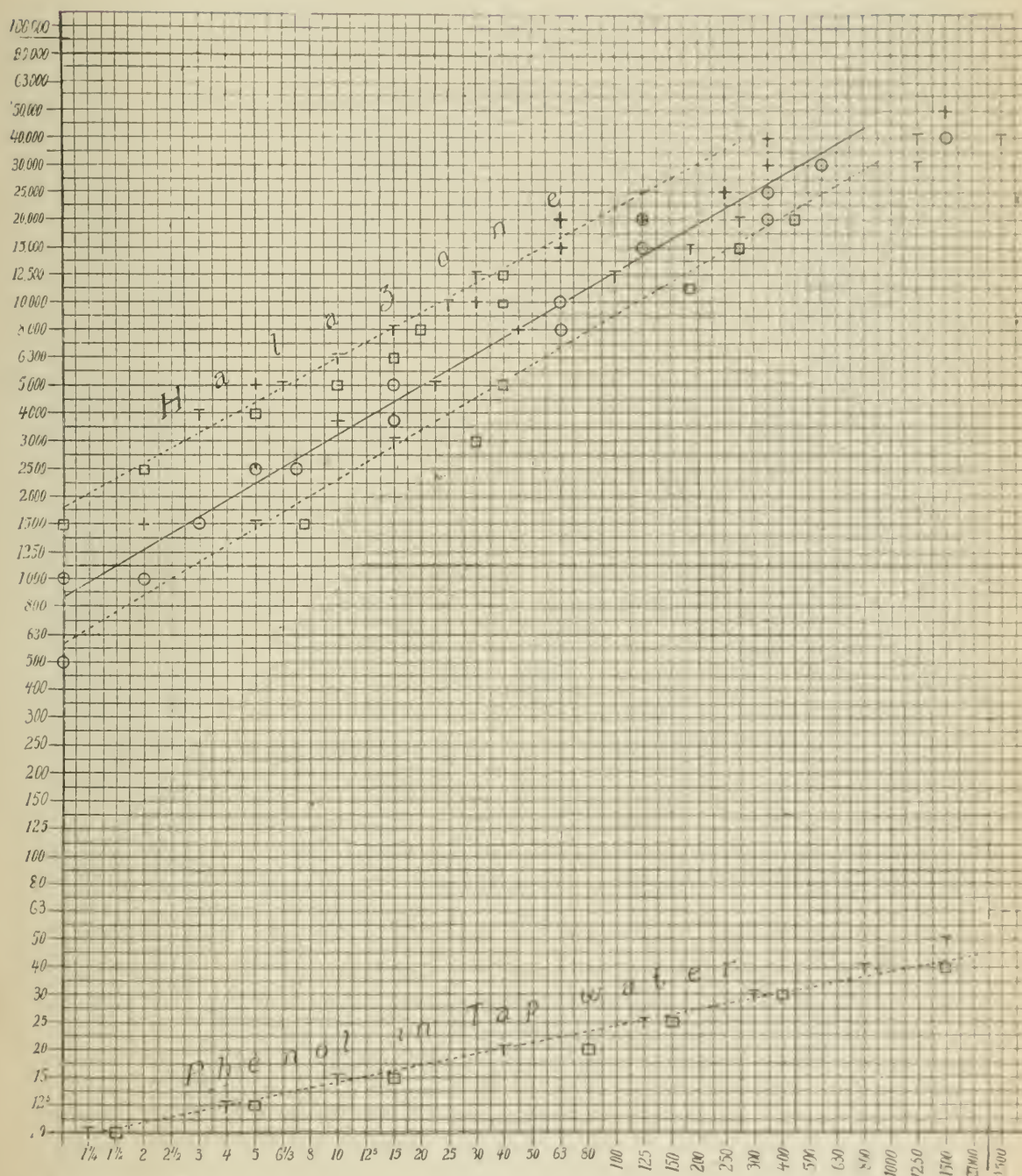


Chart 2.—Comparative results of the experiments made with halazone and phenol.

It will be noted by reference to Chart 1 that the activity of phenol ranges from N/10 (approximately 1:100), which sterilizes infected distilled water in $1\frac{1}{4}$ minutes, to N/40, which requires about 24 hours for sterilization. A solution of N/50 (1:500) phenol does not sterilize even after 24 hours. At the bottom of Chart 2 is shown the result of an experiment on the action of phenol in tap water. The line is parallel to that of phenol in distilled water and merely shifted somewhat to the right, showing that a slightly longer time is required for sterilization. Thus, with N/10 phenol it was $1\frac{1}{2}$ minutes instead of $1\frac{1}{4}$; with N/15, 15 minutes were needed in tap water, as compared with 10 minutes in distilled water. It will be seen at a glance that the disinfecting power of phenol decreases rapidly with decrease in concentration.

To obtain sterilization within 1 minute, N/5,000 of chlorinated lime would have to be used. This equals approximately 7 parts of available chlorine per million. From here the unbroken line (Chart 1), showing the action of this agent in distilled water, runs almost straight to N/10,000 which requires, in our experiment, 50 minutes for sterilization of infected distilled water. From this point a change in the direction of the line takes place, so that it becomes a curve, which ends at N/63,000. A solution as dilute as N/80,000, fails to disinfect even after 24 hours. The reason for the curving of the line I am unable to give at this time. That it is neither an accident nor an artefact, that is, due to rise of temperature, I feel able to assert on the strength of sufficiently repeated and, at times, sufficiently carefully controlled experiments. It may be that the relative improvement in the rate of action with greater dilutions is due to greater ionization in the more dilute solution. When Chicago tap water infected with typhoid bacilli was used, the results were much less consistent than with distilled water; at times, the disinfection occurred more rapidly in tap water than in distilled water; at times, more slowly, as shown by the dotted lines on Chart 1. This is evidently due to variability in the composition of the Chicago water supply, due to the effect of prevailing wind and weather on the water of Lake Michigan, near the shore. The rather surprising fact that, at times, the chlorinated lime acted more rapidly in tap water than it did in distilled water, might be due to the presence of a greater number of H-ions, which have an accelerating action, as shall be shown, or may depend on the fact that from 3-5 lbs. of liquefied chlorine are added to each million gallons of water at the Chicago water works.

The line representing the action of halazone (Chart 2) starts at N/1,000 (calculated as "available chlorin"), approximately 1:15,000 of halazone by weight. This disinfects within 2 minutes. The line extends to N/40,000, which requires almost 24 hours for action. At

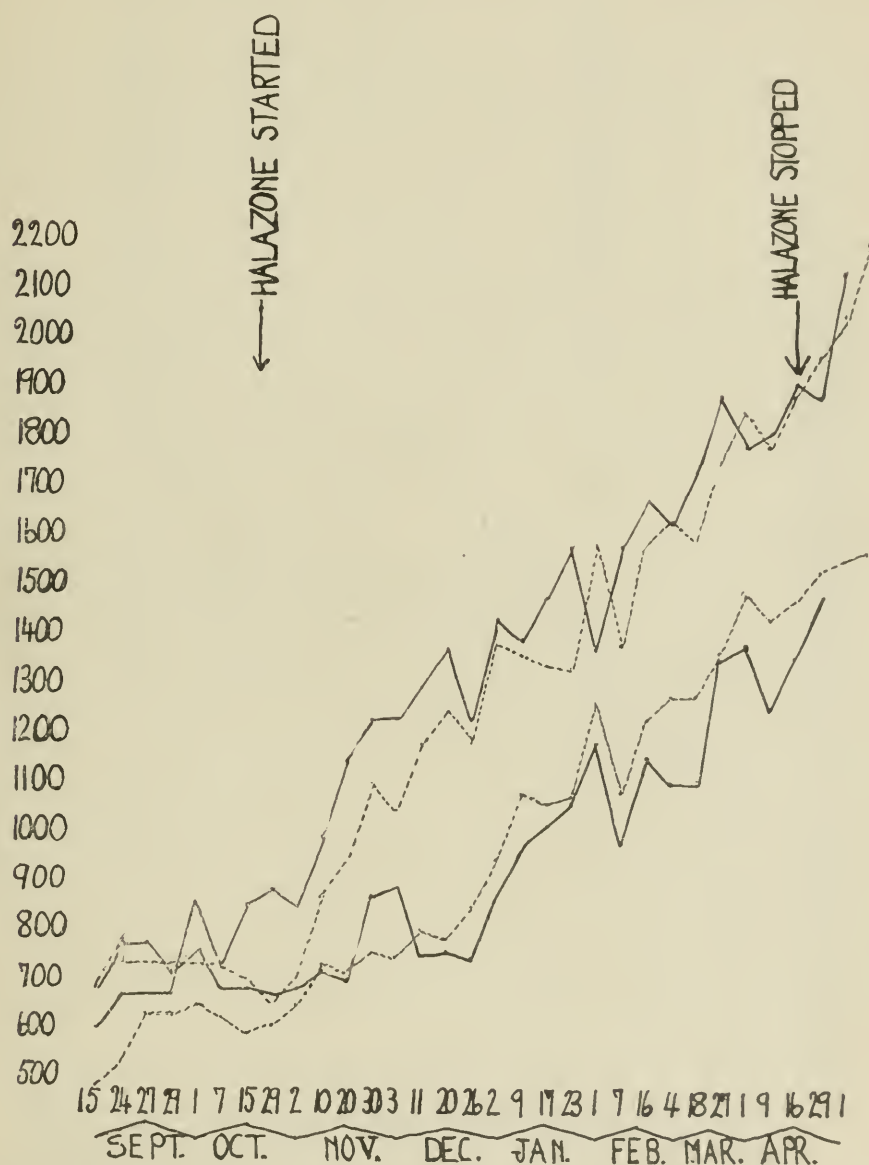


Chart 3.—Weight curve of cats given 0.1 gm. halazone daily with their food (continuous line), compared with weight curve of control animals (broken line).

N/50,000 the action ends. It should be noted, that the line is straight. This would make the theory, that the curve in the chlorinated lime line is due to ionization, agree best with the experimental facts, as halazone does not ionize. With tap water, variable results were obtained, as in case of chlorinated lime. At times, the action was

slower than in distilled water; at times, it was more rapid. Dakin and Dunham¹ found that 1:300,000 of halazone disinfected heavily polluted water within 30 minutes. This approximately equals N/20,000

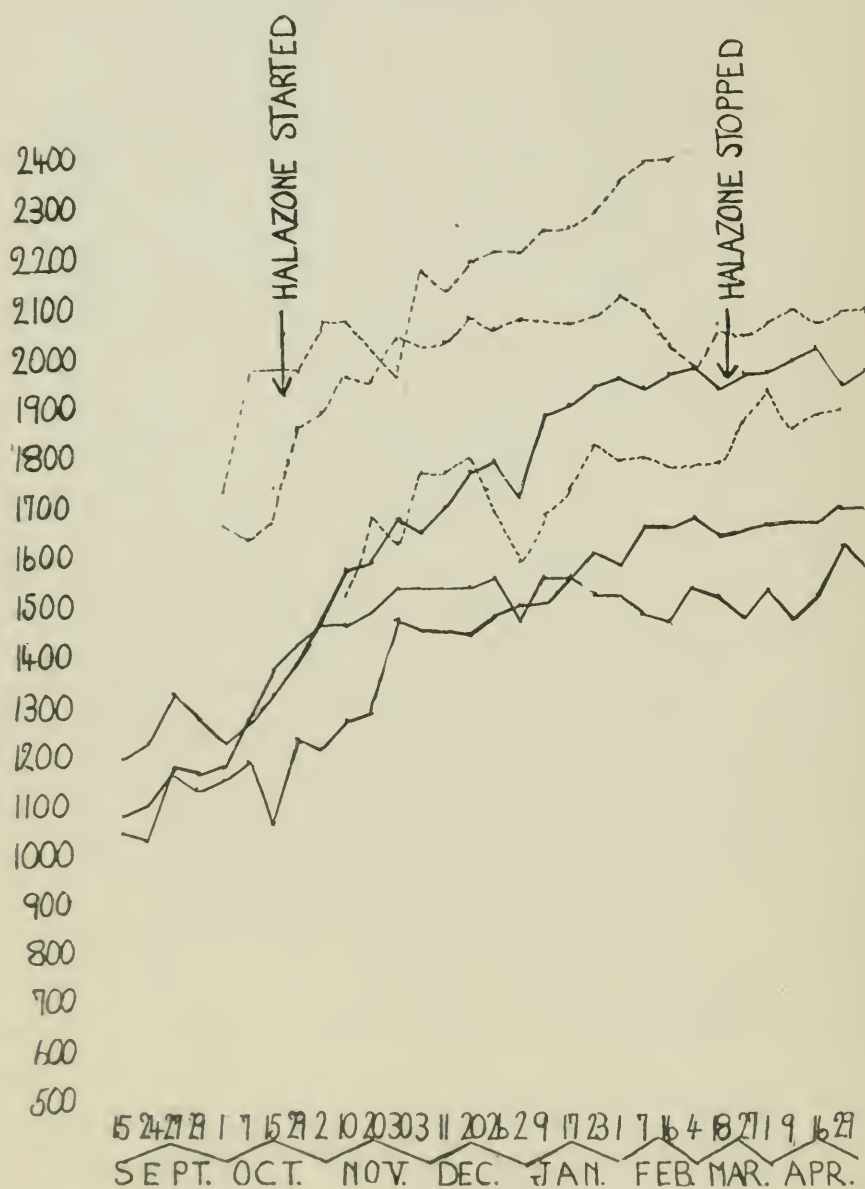


Chart 4.—Weight curve of rabbits given 0.1 gm. halazone daily (continuous line), compared with that of control animals (broken line).

halazone, which in our experiments required at an average 200 minutes in distilled water; though, in tap water, speedier as well as slower action might be obtained. This makes halazone, in the concentration recommended, appear to be a rather slow disinfectant.

To compare chlorinated lime with halazone in terms of the phenol coefficient at 20 C. for 5-20 minutes, the relation would be as follows:

Chlorinated lime	466
Halazone	228

In other words, compared on the basis of available chlorine, chlorinated lime is about twice as efficient a water disinfectant as is halazone. This superior activity is evidently due to the greater chemical instability of the chlorinated lime.

DETERMINATION OF TOXICITY

While it is well known that the products remaining after chlorinated lime has exerted its action in water disinfection are harmless, it seemed of interest to determine the toxicity of the decomposition products of halazone, most especially when fed daily for a considerable length of time. Hence, feeding experiments were undertaken, which may be described as follows:

Feeding Exper. 1.—Four kittens of one litter, of approximate similar weight, which at the beginning of the experiment ranged from 500-700 gm., were kept in separate cages and fed weighed quantities of food, consisting of milk mixed with cornmeal mush. Later, meat was given on alternate days. The quantity of food was so adjusted that the animals would empty their dishes; and, of course, was uniform for all 4 animals. After a preliminary period of 6 weeks, 2 of the animals were given halazone, while 2 were kept as controls. For medication of the food, a mixture of 0.1 gm. of halazone and 0.1 gm. of anhydrous sodium carbonate was added daily to the food. The animals consumed this medicated food almost as well as did the control animals, and gained in weight to practically the same extent as the controls, as will be seen from Chart 3, which shows the weight curve of the control animals in broken lines and of the animals that received the medicated food, in continuous lines. It will be noted that the lines are practically parallel and that the control animals did no better than those that were given the halazone mixed with their food. The animals showed no symptoms of intolerance. At the conclusion of the experiment, the halazone animals were chloroformed and their organs examined. There was no macroscopic evidence of damage to any of the organs usually examined at necropsy. Microscopic examination of the livers and kidneys showed no changes.

Feeding Exper. 2.—Three rabbits, of approximately equal weight (1050-1225 gm.), were kept in separate cages; and fed with chopped carrots mixed with oats, in such quantity as to insure complete consumption by the animal during 24 hours. After a control period of about 1½ months, the animals were given 0.1 gm. halazone and 0.1 gm. anhydrous sodium carbonate daily, mixed with their food, which they consumed quite as readily as they did the unmedicated food previously administered. Three control animals, also kept in separate cages, were fed the same amount of oats and carrots. Chart 4, which illustrates the result of this experiment, shows that the gain in weight of all the 6 animals

was sufficiently parallel to enable one to say that the halazone addition to the food evidently did not interfere with the growth and development of the rabbits that received it. The weight of 2 of the control rabbits was excessive at the beginning of the experiment, as compared with the weight of the others; the rate of their gain in weight, however, was quite proportional to that of the halazone animals. The necropsy of the halazone animals showed nothing abnormal.

Exper. 3.—Acute Poisoning. Cat 1, weight 2,700 gm., was given 2.7 gm. each of halazone and sodium carbonate dissolved in water (1 gm. per kg.) by stomach tube. Almost immediate and repeated profuse emesis was the only symptom noted. The animal recovered.

Cat 2, weighing 3,200 gm., was given 6.4 gm. of halazone (2 gm. per kg.) by means of a stomach tube. Profuse emesis, setting in within 4 minutes and repeated 3 times during the course of the next few hours, was the only result. The animal recovered.

Cat 4, weight 1,600 gm. was given 3.2 gm. of halazone (2 gm. per kg.) per stomach tube. Emesis set in 10 minutes later, and continued at intervals throughout the day. At first the vomitus consisted of food mixed with the injected material, later it was mucous. The animal died on the 2nd day after the administration of the dose; and showed, on necropsy, evidence of violent irritation of the stomach. Otherwise the organs seemed negative, though microscopic examination was not made, as the necropsy was performed too late to obtain satisfactory results from such examination.

DISCUSSION OF THE FEEDING EXPERIMENTS

The fact that the halazone was administered mixed with the food in the feeding experiments makes it evident that the chlorin was to a great extent lost by decomposition of the chloramin before the animal could eat the food. It was, therefore, chiefly p-sulphonamino-benzoic acid that the animals ingested. This probably accounts for the lack of irritation of the stomach from consumption of this food. Inasmuch as the sulphonadichloraminobenzoic acid (halazone), when used for water disinfection, is almost wholly decomposed in a similar manner before the disinfected water is ingested, the feeding experiments may be considered to show that such water could not have any deleterious effects on the human organism, especially when it is realized that 0.1 gm. per kg. would be equivalent to the ingestion of 6 gm. of halazone by a man weighing 60 kg. Such quantity of halazone would be sufficient to disinfect 600 liters of polluted water.

The experiments on acute poisoning show that large doses of halazone, whether taken in suspension or in solution by the aid of sodium carbonate, acts as a violent gastric irritant, which by reason of the prompt emesis that it provokes would hardly be likely to produce death in a human being.

TABLET-MAKING QUALITIES

Inasmuch as for the disinfection of small quantities of drinking water, such as those that might be gathered and carried by rapidly moving troops, tablets constitute by far the most satisfactory form for use, the study of the suitability of these agents for tablet-making becomes of special importance.

As lime in the form of chlorinated lime is the cheapest vehicle for chlorin and it proved itself in our studies the most efficient disinfectant, the question of the preparation of chlorinated lime tablets was taken up. I am indebted to Dr. F. O. Tonney, director of the Municipal Laboratories of the Chicago Health Department, for permission to publish the data obtained by Mr. Jay Kaplan in an inquiry, taken up in 1916, for the purpose of devising water disinfecting tablets.

The tablets were prepared by slightly moistening chlorinated lime in a mortar to make a thick paste. This was pressed in the perforations of a tablet triturate mold of vulcanite. After drying by placing the mold for from 5-10 minutes in an oven at 40-50 C., the tablets were carefully forced out by fitting the 2 parts of the mold together and applying slow pressure. The tablet triturates thus prepared were shaken into bottle and tightly stoppered. Tests showed that, under average conditions, the disinfectant retained its potency for about 4 weeks. In four months a deterioration of about 40% had occurred. Seven and one-half months later, the tablets were found to be moist and sticky so that they did not retain their shape; and they had undergone a deterioration of 82.5%. Hence, it may be concluded that chlorinated lime tablets, prepared by the tablet triturate process, do not possess satisfactory keeping qualities.

On the assumption that the moisture employed in the preparation of the tablet triturates just described was responsible for the rapid deterioration of the tablets, I had some tablets prepared by means of compression, using the following formula:

Chlorinated lime, 30%, or proportionately larger amounts of weaker lime, 0.59 gm. Sodium chlorid (granular), 10.0 gm. Divide into 100 tablets weighing 105 mg., avoiding use of lubricant.

One of these tablets will disinfect 1 liter of moderately infected water within 1 hour in cool, or much sooner in hot weather.

These tablets, made May 3, 1918, without special care as to drying, and kept in the dark in a well stoppered bottle, were assayed from time to time with the following results:

May 6, 1918: 5 tablets weighing 0.538 gm. yielded 9.2 mg. available Cl.

June 15, 1918: 5 tablets weighing 0.544 gm. yielded 8.1 mg. available Cl.

July 8, 1918: 5 tablets weighing 0.529 gm. yielded 7.1 mg. available Cl.

Aug. 9, 1918, 5 tablets weighing 0.536 gm. yielded 6.5 mg. available Cl.

Sept. 6, 1918: 5 tablets weighing 0.533 gm. yielded 5 mg. available Cl.

This is equivalent to a deterioration of about 10% per month. Had the tablets been prepared from dried material, the deterioration would, no doubt, have been slower. Nevertheless, it must be admitted that chlorinated lime tablets are decidedly unstable. However, by the time the deterioration has reached 50%, which might be in the course of half a year, these tablets would still be as good as ever for disinfecting half the quantity of water.

It is, indeed, this very unstability of chlorinated lime tablets that is the *raison d'être* for halazone. Dakin and Dunham propose the following formula for these tablets:

Halazone	4 gm.
Sodium carbonate (dried)	4 gm.
Sodium chlorid	92 gm.

The material should be carefully dried and mixed, the alkali being added last, and made into 100 mg. tablets, without use of lubricant. They claim that 1 such tablet is capable of disinfecting 1 liter of water in 30-60 minutes. In our experiments, still longer time was required for disinfection.

Commercial halazone tablets showed the following results on assay from time to time:

Bottle opened May 6, 1918.

May 6: 5 tablets, weighing 0.550 yield 10.6 mg. available Cl.

July 8: 5 tablets, weighing 0.561, yield 10.6 mg. available Cl.

Aug. 9: 5 tablets, weighing 0.573, yield 10.6 mg. available Cl.

Sept. 6: 5 tablets, weighing 0.577, yield 11.2 mg. available Cl.

Therefore, there had been practically no deterioration in six months.

CONCLUSIONS

Chlorinated lime is a more efficient water disinfectant than halazone, compared on the basis of active chlorin. It is, of course, also a great deal cheaper. Hence there is no reason for employing halazone in water disinfection, excepting when its superior tablet-making qualities render its use advantageous.

Halazone is the better agent for the preparation of water disinfecting tablets. Its action is, however, a rather slow one.

FURTHER OBSERVATIONS ON ANTIGENS USED IN THE WASSERMANN REACTION

E. H. RUEDIGER

From the Pathological Laboratory of the Bismarck Hospital, Bismarck, N. D.

Among the antigens commonly recommended for the Wassermann reaction one finds alcoholic extract of dog heart muscle and alcoholic extract of guinea-pig heart muscle. Kolmer¹ in discussing the preparation of alcoholic extracts of normal organs says human, guinea-pig, and beef-heart muscle are usually employed.

In a previous report² I showed that with alcoholic extract of dog-heart muscle one obtains many positive results on serums which give negative results with alcoholic extract of human-heart muscle and in persons who show no signs nor symptoms of syphilis.

In the tests here reported on the serums of 50 supposedly non-syphilitic persons, and of 13 known syphilitics 6 different antigens were used: alcoholic extract of human-heart muscle (A. E. H. H.); alcoholic extract of beef-heart muscle (A. E. B. H.); alcoholic extract of dog-heart muscle (A. E. D. H.); alcoholic extract of guinea-pig-heart muscle (A. E. G. P. H.); alcoholic extract of rabbit-heart muscle (A. E. R. H.), and alcoholic extract of sheep-heart muscle (A. E. S. H.). In these tests the antigen dilutions were a little lower than in those previously reported, otherwise the technic was identical.

On a small number of human serums different dilutions of the same antigen were tested, and in the third test the antigen was added to the heated human serum 15 minutes after and 15 minutes before the complement was added.

Table 1 shows the results given by the 50 serums from clinically nonsyphilitic persons and by 13 from clinically syphilitic persons. The alcoholic extract of human heart, alcoholic extract of beef heart, alcoholic extract of rabbit heart and alcoholic extract of sheep heart gave negative results on all of the 50 nonsyphilitic cases while the alcoholic extract of dog heart gave positive results with 16 (32%), and the alcoholic extract of guinea-pig heart gave positive results with

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¹ *Infection, Immunity and Specific Therapy*, 1917, p. 445.

² *Jour. Infect. Dis.*, 1919, 4, p. 31.

TABLE 1
COMPARISON OF ANTIGENS

Number of Serum	Kind of Antigen	Dilution of Antigen	Amboceptor per Tube, Unit	Readings*						Results	
				Antigen Tubes			Control Tubes				
				1	2	3	1'	2'	3'		
1	A. E. H. H.	1:25	1 g	+	+	±	+	+	±	Negative, Negative, Negative, Negative, Negative,	—
	A. E. B. H.	1:25	1 g	+	+	±	+	+	±		
	A. E. D. H.	1:30	1 g	+	+	±	+	+	±		
	A. E. G-P. H.	1:30	1 g	+	+	±	+	+	±		
	A. E. R. H.	1:25	1 g	+	+	±	+	+	±		
2	A. E. S. H.	1:25	1 g	+	+	±	+	+	±	Negative, Negative, Negative, Negative, Negative,	—
	A. E. H. H.	1:25	1 g	+	+	±	+	+	±		
	A. E. B. H.	1:25	1 g	+	+	±	+	+	±		
	A. E. D. H.	1:30	1 g	+	+	±	+	+	±		
	A. E. G-P. H.	1:30	1 g	+	+	±	+	+	±		
3	A. E. R. H.	1:25	1 g	+	+	±	+	+	±	Negative, Negative, Negative, Negative, Negative,	—
	A. E. S. H.	1:25	1 g	+	+	±	+	+	±		
	A. E. H. H.	1:25	1 g	+	+	±	+	+	±		
	A. E. B. H.	1:25	1 g	+	+	±	+	+	±		
	A. E. D. H.	1:30	1 g	+	+	±	+	+	±		
4	A. E. G-P. H.	1:30	1 g	+	+	±	+	+	±	Negative, Negative, Negative, Negative, Negative,	—
	A. E. R. H.	1:25	1 g	+	+	±	+	+	±		
	A. E. S. H.	1:25	1 g	+	+	±	+	+	±		
	A. E. H. H.	1:25	1 g	+	+	±	+	+	±		
	A. E. B. H.	1:25	1 g	+	+	±	+	+	±		
5	A. E. D. H.	1:30	1 g	+	+	±	+	+	±	Negative, Negative, Negative, Negative, Negative,	—
	A. E. G-P. H.	1:30	1 g	+	+	±	+	+	±		
	A. E. R. H.	1:25	1 g	+	+	±	+	+	±		
	A. E. S. H.	1:25	1 g	+	+	±	+	+	±		
	A. E. H. H.	1:25	1 g	+	+	±	+	+	±		
6	A. E. B. H.	1:25	1 g	+	+	±	+	+	±	Negative, Negative, Negative, Negative, Negative,	—
	A. E. D. H.	1:30	1 g	+	+	±	+	+	±		
	A. E. G-P. H.	1:30	1 g	+	+	±	+	+	±		
	A. E. R. H.	1:25	1 g	+	+	±	+	+	±		
	A. E. S. H.	1:25	1 g	+	+	±	+	+	±		
7	A. E. H. H.	1:25	1 g	+	+	±	+	+	±	Negative, Negative, Negative, Negative, Negative,	—
	A. E. B. H.	1:25	1 g	+	+	±	+	+	±		
	A. E. D. H.	1:30	1 g	+	+	±	+	+	±		
	A. E. G-P. H.	1:30	1 g	+	+	±	+	+	±		
	A. E. R. H.	1:25	1 g	+	+	±	+	+	±		
8	A. E. S. H.	1:25	1 g	+	+	±	+	+	±	Negative, Negative, Negative, Negative, Negative,	—
	A. E. H. H.	1:25	1 g	+	+	±	+	+	±		
	A. E. B. H.	1:25	1 g	+	+	±	+	+	±		
	A. E. D. H.	1:30	1 g	+	+	±	+	+	±		
	A. E. G-P. H.	1:30	1 g	+	+	±	+	+	±		
9	A. E. R. H.	1:25	1 g	+	+	±	+	+	±	Negative, Negative, Negative, Negative, Negative,	—
	A. E. S. H.	1:25	1 g	+	+	±	+	+	±		
	A. E. H. H.	1:25	1 g	+	+	±	+	+	±		
	A. E. B. H.	1:25	1 g	+	+	±	+	+	±		
	A. E. D. H.	1:30	1 g	+	+	±	+	+	±		

* Explanation: + means complete hemolysis; ±, hemolysis between 50% and 100%; tr (trace), hemolysis less than 50%; 0, no hemolysis.

TABLE 1—Continued
COMPARISON OF ANTIGENS

Number of Serum	Kind of Antigen	Dilution of Antigen	Amboceptor per Tube, Unit	Readings*						Results
				Antigen Tubes			Control Tubes			
				1	2	3	1'	2'	3'	
10	A. E. H. H.	1:25	1 g	+	+	±	+	+	±	Negative, Negative, Negative, Negative, Negative, Negative,
	A. E. B. H.	1:25	1 g	+	+	±	+	+	±	
	A. E. D. H.	1:30	1 g	+	+	±	+	+	±	
	A. E. G-P. H.	1:30	1 g	+	+	±	+	+	±	
	A. E. R. H.	1:25	1 g	+	+	±	+	+	±	
	A. E. S. H.	1:25	1 g	+	+	±	+	+	±	
11	A. E. H. H.	1:25	1 g	+	+	±	+	+	±	Negative, Negative, Negative, Negative, Negative, Negative,
	A. E. B. H.	1:25	1 g	+	+	±	+	+	±	
	A. E. D. H.	1:30	1 g	+	+	±	+	+	±	
	A. E. G-P. H.	1:30	1 g	+	+	±	+	+	±	
	A. E. R. H.	1:25	1 g	+	+	±	+	+	±	
	A. E. S. H.	1:25	1 g	+	+	±	+	+	±	
12	A. E. H. H.	1:25	1 g	+	+	±	+	+	±	Negative, Negative, Negative, Negative, Negative, Negative,
	A. E. B. H.	1:25	1 g	+	+	±	+	+	±	
	A. E. D. H.	1:30	1 g	+	+	±	+	+	±	
	A. E. G-P. H.	1:30	1 g	+	+	±	+	+	±	
	A. E. R. H.	1:25	1 g	+	+	±	+	+	±	
	A. E. S. H.	1:25	1 g	+	+	±	+	+	±	
13	A. E. H. H.	1:25	1 g	+	+	tr	+	+	tr	Negative, Negative, Negative, Negative, Negative, Negative,
	A. E. B. H.	1:25	1 g	+	+	tr	+	+	tr	
	A. E. D. H.	1:30	1 g	+	+	tr	+	+	tr	
	A. E. G-P. H.	1:30	1 g	+	+	tr	+	+	tr	
	A. E. R. H.	1:25	1 g	+	+	tr	+	+	tr	
	A. E. S. H.	1:25	1 g	+	+	tr	+	+	tr	
14	A. E. H. H.	1:25	1 g	+	+	±	+	+	±	Negative, Negative, Negative, Negative, Negative, Negative,
	A. E. B. H.	1:25	1 g	+	+	±	+	+	±	
	A. E. D. H.	1:30	1 g	+	+	±	+	+	±	
	A. E. G-P. H.	1:30	1 g	+	+	±	+	+	±	
	A. E. R. H.	1:25	1 g	+	+	±	+	+	±	
	A. E. S. H.	1:25	1 g	+	+	±	+	+	±	
15	A. E. H. H.	1:25	1 g	+	+	±	+	+	±	Negative, Negative, Negative, Negative, Negative, Negative,
	A. E. B. H.	1:25	1 g	+	+	±	+	+	±	
	A. E. D. H.	1:30	1 g	+	+	±	+	+	±	
	A. E. G-P. H.	1:30	1 g	+	+	±	+	+	±	
	A. E. R. H.	1:25	1 g	+	+	±	+	+	±	
	A. E. S. H.	1:25	1 g	+	+	±	+	+	±	
16	A. E. H. H.	1:25	1 g	+	+	tr	+	+	tr	Negative, Negative, Negative, Negative, Negative, Negative,
	A. E. B. H.	1:25	1 g	+	+	tr	+	+	tr	
	A. E. D. H.	1:30	1 g	+	+	tr	+	+	tr	
	A. E. G-P. H.	1:30	1 g	+	+	tr	+	+	tr	
	A. E. R. H.	1:25	1 g	+	+	tr	+	+	tr	
	A. E. S. H.	1:25	1 g	+	+	tr	+	+	tr	
17	A. E. H. H.	1:25	1 g	+	+	tr	+	+	tr	Negative, Negative, Negative, Negative, Negative, Negative,
	A. E. B. H.	1:25	1 g	+	+	tr	+	+	tr	
	A. E. D. H.	1:30	1 g	+	+	tr	+	+	tr	
	A. E. G-P. H.	1:30	1 g	+	+	tr	+	+	tr	
	A. E. R. H.	1:25	1 g	+	+	tr	+	+	tr	
	A. E. S. H.	1:25	1 g	+	+	tr	+	+	tr	
18	A. E. H. H.	1:25	1 g	+	+	±	+	+	±	Negative, Negative, Negative, Negative, Negative, Negative,
	A. E. B. H.	1:25	1 g	+	+	±	+	+	±	
	A. E. D. H.	1:30	1 g	+	+	±	+	+	±	
	A. E. G-P. H.	1:30	1 g	+	+	±	+	+	±	
	A. E. R. H.	1:25	1 g	+	+	±	+	+	±	
	A. E. S. H.	1:25	1 g	+	+	±	+	+	±	

TABLE 1—Continued
COMPARISON OF ANTIGENS

Number of Serum	Kind of Antigen	Dilution of Antigen	Amboceptor per Tube, Unit	Readings*						Results	
				Antigen Tubes			Control Tubes				
				1	2	3	1'	2'	3'		
19	A. E. H. H.	1:25	1 g	+	+	±	+	+	±	Negative,	—
	A. E. B. H.	1:25	1 g	+	+	±	+	+	±	Negative,	—
	A. E. D. H.	1:30	1 g	+	+	±	+	+	±	Negative,	—
	A. E. G-P. H.	1:30	1 g	+	+	±	+	+	±	Negative,	—
	A. E. R. H.	1:25	1 g	+	+	±	+	+	±	Negative,	—
	A. E. S. H.	1:25	1 g	+	+	±	+	+	±	Negative,	—
20	A. E. H. H.	1:25	1 g	+	+	±	+	+	±	Negative,	—
	A. E. B. H.	1:25	1 g	+	+	±	+	+	±	Negative,	—
	A. E. D. H.	1:30	1 g	+	+	±	+	+	±	Negative,	—
	A. E. G-P. H.	1:30	1 g	+	+	±	+	+	±	Negative,	—
	A. E. R. H.	1:25	1 g	+	+	±	+	+	±	Negative,	—
	A. E. S. H.	1:25	1 g	+	+	±	+	+	±	Negative,	—
21	A. E. H. H.	1:25	1 g	+	+	±	+	+	±	Negative,	—
	A. E. B. H.	1:25	1 g	+	+	±	+	+	±	Negative,	—
	A. E. D. H.	1:30	1 g	+	+	±	+	+	±	Negative,	—
	A. E. G-P. H.	1:30	1 g	+	+	±	+	+	±	Negative,	—
	A. E. R. H.	1:25	1 g	+	+	±	+	+	±	Negative,	—
	A. E. S. H.	1:25	1 g	+	+	±	+	+	±	Negative,	—
22	A. E. H. H.	1:25	1 g	+	+	±	+	+	±	Negative,	—
	A. E. B. H.	1:25	1 g	+	+	±	+	+	±	Negative,	—
	A. E. D. H.	1:30	1 g	+	+	±	+	+	±	Negative,	—
	A. E. G-P. H.	1:30	1 g	+	+	±	+	+	±	Negative,	—
	A. E. R. H.	1:25	1 g	+	+	±	+	+	±	Negative,	—
	A. E. S. H.	1:25	1 g	+	+	±	+	+	±	Negative,	—
23	A. E. H. H.	1:25	1 g	+	+	±	+	+	±	Negative,	—
	A. E. B. H.	1:25	1 g	+	+	±	+	+	±	Negative,	—
	A. E. D. H.	1:30	1 g	+	+	±	+	+	±	Negative,	—
	A. E. G-P. H.	1:30	1 g	+	+	±	+	+	±	Negative,	—
	A. E. R. H.	1:25	1 g	+	+	±	+	+	±	Negative,	—
	A. E. S. H.	1:25	1 g	+	+	±	+	+	±	Negative,	—
24	A. E. H. H.	1:25	1 g	+	+	tr	+	+	tr	Negative,	—
	A. E. B. H.	1:25	1 g	+	+	tr	+	+	tr	Negative,	—
	A. E. D. H.	1:30	1 g	+	+	tr	+	+	tr	Negative,	—
	A. E. G-P. H.	1:30	1 g	+	+	tr	+	+	tr	Negative,	—
	A. E. R. H.	1:25	1 g	+	+	tr	+	+	tr	Negative,	—
	A. E. S. H.	1:25	1 g	+	+	tr	+	+	tr	Negative,	—
25	A. E. H. H.	1:25	1 g	+	+	±	+	+	±	Negative,	—
	A. E. B. H.	1:25	1 g	+	+	±	+	+	±	Negative,	—
	A. E. D. H.	1:30	1 g	+	+	±	+	+	±	Negative,	—
	A. E. G-P. H.	1:30	1 g	+	+	±	+	+	±	Negative,	—
	A. E. R. H.	1:25	1 g	+	+	±	+	+	±	Negative,	—
	A. E. S. H.	1:25	1 g	+	+	±	+	+	±	Negative,	—
26	A. E. H. H.	1:25	1 g	+	+	±	+	+	±	Negative,	—
	A. E. B. H.	1:25	1 g	+	+	±	+	+	±	Negative,	—
	A. E. D. H.	1:30	1 g	+	+	±	+	+	±	Negative,	—
	A. E. G-P. H.	1:30	1 g	+	+	±	+	+	±	Negative,	—
	A. E. R. H.	1:25	1 g	+	+	±	+	+	±	Negative,	—
	A. E. S. H.	1:25	1 g	+	+	±	+	+	±	Negative,	—
27	A. E. H. H.	1:25	1 g	+	+	±	+	+	±	Negative,	—
	A. E. B. H.	1:25	1 g	+	+	±	+	+	±	Negative,	—
	A. E. D. H.	1:30	1 g	+	+	±	+	+	±	Negative,	—
	A. E. G-P. H.	1:30	1 g	+	+	±	+	+	±	Negative,	—
	A. E. R. H.	1:25	1 g	+	+	±	+	+	±	Negative,	—
	A. E. S. H.	1:25	1 g	+	+	±	+	+	±	Negative,	—

TABLE 1—Continued
COMPARISON OF ANTIGENS

Number of Serum	Kind of Antigen	Dilution of Antigen	Ambo- ceptor per Tube, Unit	Readings*						Results	
				Antigen Tubes			Control Tubes				
				1	2	3	1'	2'	3'		
28	A. E. H. H.	1:25	1 g	+	+	±	+	+	±	Negative,	—
	A. E. B. H.	1:25	1 g	+	+	±	+	+	±	Negative,	—
	A. E. D. H.	1:30	1 g	+	+	±	+	+	±	Negative,	—
	A. E. G-P. H.	1:30	1 g	+	+	±	+	+	±	Negative,	—
	A. E. R. H.	1:25	1 g	+	+	±	+	+	±	Negative,	—
	A. E. S. H.	1:25	1 g	+	+	±	+	+	±	Negative,	—
29	A. E. H. H.	1:25	1 g	+	+	±	+	+	±	Negative,	—
	A. E. B. H.	1:25	1 g	+	+	±	+	+	±	Negative,	—
	A. E. D. H.	1:30	1 g	+	+	±	+	+	±	Negative,	—
	A. E. G-P. H.	1:30	1 g	+	+	±	+	+	±	Negative,	—
	A. E. R. H.	1:25	1 g	+	+	±	+	+	±	Negative,	—
	A. E. S. H.	1:25	1 g	+	+	±	+	+	±	Negative,	—
30	A. E. H. H.	1:25	1 g	+	+	±	+	+	±	Negative,	—
	A. E. B. H.	1:25	1 g	+	+	±	+	+	±	Negative,	—
	A. E. D. H.	1:30	1 g	+	+	±	+	+	±	Negative,	—
	A. E. G-P. H.	1:30	1 g	+	+	±	+	+	±	Negative,	—
	A. E. R. H.	1:25	1 g	+	+	±	+	+	±	Negative,	—
	A. E. S. H.	1:25	1 g	+	+	±	+	+	±	Negative,	—
31	A. E. H. H.	1:25	1 g	+	+	±	+	+	±	Negative,	—
	A. E. B. H.	1:25	1 g	+	+	±	+	+	±	Negative,	—
	A. E. D. H.	1:30	1 g	+	+	±	+	+	±	Negative,	—
	A. E. G-P. H.	1:30	1 g	+	+	±	+	+	±	Negative,	—
	A. E. R. H.	1:25	1 g	+	+	±	+	+	±	Negative,	—
	A. E. S. H.	1:25	1 g	+	+	±	+	+	±	Negative,	—
32	A. E. H. H.	1:25	1 g	+	+	tr	+	+	tr	Negative,	—
	A. E. B. H.	1:25	1 g	+	+	tr	+	+	tr	Negative,	—
	A. E. D. H.	1:30	1 g	+	+	tr	+	+	tr	Negative,	—
	A. E. G-P. H.	1:30	1 g	+	+	tr	+	+	tr	Negative,	—
	A. E. R. H.	1:25	1 g	+	+	tr	+	+	tr	Negative,	—
	A. E. S. H.	1:25	1 g	+	+	tr	+	+	tr	Negative,	—
33	A. E. H. H.	1:25	1 g	+	+	±	+	+	±	Negative,	—
	A. E. B. H.	1:25	1 g	+	+	±	+	+	±	Negative,	—
	A. E. D. H.	1:30	1 g	+	+	±	+	+	±	Negative,	—
	A. E. G-P. H.	1:30	1 g	+	+	±	+	+	±	Negative,	—
	A. E. R. H.	1:25	1 g	+	+	±	+	+	±	Negative,	—
	A. E. S. H.	1:25	1 g	+	+	±	+	+	±	Negative,	—
34	A. E. H. H.	1:25	1 g	+	+	±	+	+	±	Negative,	—
	A. E. B. H.	1:25	1 g	+	+	±	+	+	±	Negative,	—
	A. E. D. H.	1:30	1 g	+	+	0	+	+	±	Moderately positive, 2+	—
	A. E. G-P. H.	1:30	1 g	+	tr	0	+	+	±	Strongly positive, 4+	—
	A. E. R. H.	1:25	1 g	+	+	±	+	+	±	Negative,	—
	A. E. S. H.	1:25	1 g	+	+	±	+	+	±	Negative,	—
35	A. E. H. H.	1:25	1 g	+	+	±	+	+	±	Negative,	—
	A. E. B. H.	1:25	1 g	+	+	±	+	+	±	Negative,	—
	A. E. D. H.	1:30	1 g	+	0	0	+	+	±	Strongly positive, 5+	—
	A. E. G-P. H.	1:30	1 g	+	0	0	+	+	±	Strongly positive, 5+	—
	A. E. R. H.	1:25	1 g	+	+	±	+	+	±	Negative,	—
	A. E. S. H.	1:25	1 g	+	+	±	+	+	±	Negative,	—
36	A. E. H. H.	1:25	1 g	+	+	±	+	+	±	Negative,	—
	A. E. B. H.	1:25	1 g	+	+	±	+	+	±	Negative,	—
	A. E. D. H.	1:30	1 g	+	+	0	+	+	±	Strongly positive, 3+	—
	A. E. G-P. H.	1:30	1 g	+	tr	0	+	+	±	Strongly positive, 4+	—
	A. E. R. H.	1:25	1 g	+	+	±	+	+	±	Negative,	—
	A. E. S. H.	1:25	1 g	+	+	±	+	+	±	Negative,	—

TABLE 1—Continued
COMPARISON OF ANTIGENS

Number of Serum	Kind of Antigen	Dilution of Antigen	Amboceptor per Tube, Unit	Readings*						Results	
				Antigen Tubes			Control Tubes				
				1	2	3	1'	2'	3'		
37	A. E. H. H.	1:25	1 g	+	+	±	+	+	±	Negative,	—
	A. E. B. H.	1:25	1 g	+	+	±	+	+	±	Negative,	—
	A. E. D. H.	1:30	1 g	+	+	±	+	+	±	Negative,	—
	A. E. G-P. H.	1:30	1 g	+	+	tr	+	+	±	Weakly positive,	—
	A. E. R. H.	1:25	1 g	+	+	±	+	+	±	Negative,	—
	A. E. S. H.	1:25	1 g	+	+	±	+	+	±	Negative,	—
38	A. E. H. H.	1:25	1 g	+	+	±	+	+	±	Negative,	—
	A. E. B. H.	1:25	1 g	+	+	±	+	+	±	Negative,	—
	A. E. D. H.	1:30	1 g	+	+	tr	+	+	±	Weakly positive,	1+
	A. E. G-P. H.	1:30	1 g	+	±	0	+	+	±	Strongly positive,	3+
	A. E. R. H.	1:25	1 g	+	+	±	+	+	±	Negative,	—
	A. E. S. H.	1:25	1 g	+	+	±	+	+	±	Negative,	—
39	A. E. H. H.	1:25	1 g	+	+	±	+	+	±	Negative,	—
	A. E. B. H.	1:25	1 g	+	+	±	+	+	±	Negative,	—
	A. E. D. H.	1:30	1 g	+	+	0	+	+	±	Moderately positive,	2+
	A. E. G-P. H.	1:30	1 g	+	0	0	+	+	±	Strongly positive,	5+
	A. E. R. H.	1:25	1 g	+	+	±	+	+	±	Negative,	—
	A. E. S. H.	1:25	1 g	+	+	±	+	+	±	Negative,	—
40	A. E. H. H.	1:25	1 g	+	+	tr	+	+	tr	Negative,	—
	A. E. B. H.	1:25	1 g	+	+	tr	+	+	tr	Negative,	—
	A. E. D. H.	1:30	1 g	+	tr	0	+	+	tr	Strongly positive,	3+
	A. E. G-P. H.	1:30	1 g	+	0	0	+	+	tr	Strongly positive,	4+
	A. E. R. H.	1:25	1 g	+	+	tr	+	+	tr	Negative,	—
	A. E. S. H.	1:25	1 g	+	+	tr	+	+	tr	Negative,	—
41	A. E. H. H.	1:25	1 g	+	+	±	+	+	±	Negative,	—
	A. E. B. H.	1:25	1 g	+	+	±	+	+	±	Negative,	—
	A. E. D. H.	1:30	1 g	+	+	tr	+	+	±	Weakly positive,	1+
	A. E. G-P. H.	1:30	1 g	+	+	0	+	+	±	Moderately positive,	2+
	A. E. R. H.	1:25	1 g	+	+	±	+	+	±	Negative,	—
	A. E. S. H.	1:25	1 g	+	+	±	+	+	±	Negative,	—
42	A. E. H. H.	1:25	1 g	+	+	±	+	+	±	Negative,	—
	A. E. B. H.	1:25	1 g	+	+	±	+	+	±	Negative,	—
	A. E. D. H.	1:30	1 g	+	+	tr	+	+	±	Weakly positive,	1+
	A. E. G-P. H.	1:30	1 g	+	+	0	+	+	±	Moderately positive,	2+
	A. E. R. H.	1:25	1 g	+	+	±	+	+	±	Negative,	—
	A. E. S. H.	1:25	1 g	+	+	±	+	+	±	Negative,	—
43	A. E. H. H.	1:25	1 g	+	+	±	+	+	±	Negative,	—
	A. E. B. H.	1:25	1 g	+	+	±	+	+	±	Negative,	—
	A. E. D. H.	1:30	1 g	+	+	0	+	+	±	Moderately positive,	2+
	A. E. G-P. H.	1:30	1 g	+	±	0	+	+	±	Strongly positive,	3+
	A. E. R. H.	1:25	1 g	+	+	±	+	+	±	Negative,	—
	A. E. S. H.	1:25	1 g	+	+	±	+	+	±	Negative,	—
44	A. E. H. H.	1:25	1 g	+	+	±	+	+	±	Negative,	—
	A. E. B. H.	1:25	1 g	+	+	±	+	+	±	Negative,	—
	A. E. D. H.	1:30	1 g	+	±	0	+	+	±	Strongly positive,	3+
	A. E. G-P. H.	1:30	1 g	+	tr	0	+	+	±	Strongly positive,	4+
	A. E. R. H.	1:25	1 g	+	+	±	+	+	±	Negative,	—
	A. E. S. H.	1:25	1 g	+	+	±	+	+	±	Negative,	—
45	A. E. H. H.	1:25	1 g	+	+	±	+	+	±	Negative,	—
	A. E. B. H.	1:25	1 g	+	+	±	+	+	±	Negative,	—
	A. E. D. H.	1:30	1 g	+	+	0	+	+	±	Moderately positive,	2+
	A. E. G-P. H.	1:30	1 g	+	+	0	+	+	±	Moderately positive,	2+
	A. E. R. H.	1:25	1 g	+	+	±	+	+	±	Negative,	—
	A. E. S. H.	1:25	1 g	+	+	±	+	+	±	Negative,	—

TABLE 1—Continued
COMPARISON OF ANTIGENS

Number of Serum	Kind of Antigen	Dilution of Antigen	Ambo- ceptor per Tube, Unit	Readings*						Results	
				Antigen Tubes			Control Tubes				
				1	2	3	1'	2'	3'		
46	A. E. H. H.	1:25	1 g	+	+	±	+	+	±	Negative,	—.
	A. E. B. H.	1:25	1 g	+	+	±	+	+	±	Negative,	—.
	A. E. D. H.	1:30	1 g	+	+	tr	+	+	±	Weakly positive,	1+
	A. E. G-P. H.	1:30	1 g	+	+	tr	+	+	±	Weakly positive,	1+
	A. E. R. H.	1:25	1 g	+	+	±	+	+	±	Negative,	—.
	A. E. S. H.	1:25	1 g	+	+	±	+	+	±	Negative,	—.
47	A. E. H. H.	1:25	1 g	+	+	±	+	+	±	Negative,	—.
	A. E. B. H.	1:25	1 g	+	+	±	+	+	±	Negative,	—.
	A. E. D. H.	1:30	1 g	+	+	tr	+	+	±	Weakly positive,	1+
	A. E. G-P. H.	1:30	1 g	+	+	0	+	+	±	Moderately positive,	2+
	A. E. R. H.	1:25	1 g	+	+	±	+	+	±	Negative,	—.
	A. E. S. H.	1:25	1 g	+	+	±	+	+	±	Negative,	—.
48	A. E. H. H.	1:25	1 g	+	+	tr	+	+	tr	Negative,	—.
	A. E. B. H.	1:25	1 g	+	+	tr	+	+	tr	Negative,	—.
	A. E. D. H.	1:30	1 g	+	+	0	+	+	tr	Weakly positive,	1+
	A. E. G-P. H.	1:30	1 g	+	+	0	+	+	tr	Weakly positive,	1+
	A. E. R. H.	1:25	1 g	+	+	tr	+	+	tr	Negative,	—.
	A. E. S. H.	1:25	1 g	+	+	tr	+	+	tr	Negative,	—.
49	A. E. H. H.	1:25	1 g	+	+	±	+	+	±	Negative,	—.
	A. E. B. H.	1:25	1 g	+	+	±	+	+	±	Negative,	—.
	A. E. D. H.	1:30	1 g	+	+	tr	+	+	±	Weakly positive,	1+
	A. E. G-P. H.	1:30	1 g	+	+	0	+	+	±	Moderately positive,	2+
	A. E. R. H.	1:25	1 g	+	+	±	+	+	±	Negative,	—.
	A. E. S. H.	1:25	1 g	+	+	±	+	+	±	Negative,	—.
50	A. E. H. H.	1:25	1 g	+	+	±	+	+	±	Negative,	—.
	A. E. B. H.	1:25	1 g	+	+	±	+	+	±	Negative,	—.
	A. E. D. H.	1:30	1 g	+	+	0	+	+	±	Moderately positive,	2+
	A. E. G-P. H.	1:30	1 g	0	0	0	+	+	±	Strongly positive,	10+
	A. E. R. H.	1:25	1 g	+	+	±	+	+	±	Negative,	—.
	A. E. S. H.	1:25	1 g	+	+	±	+	+	±	Negative,	—.
51	A. E. H. H.	1:25	1 g	+	+	0	+	+	±	Moderately positive,	2+
	A. E. B. H.	1:25	1 g	+	±	0	+	+	±	Strongly positive,	3+
	A. E. D. H.	1:30	1 g	+	+	0	+	+	±	Moderately positive,	2+
	A. E. G-P. H.	1:30	1 g	+	+	0	+	+	±	Moderately positive,	2+
	A. E. R. H.	1:25	1 g	+	+	0	+	+	±	Moderately positive,	2+
	A. E. S. H.	1:25	1 g	+	+	±	+	+	±	Negative,	—.
52	A. E. H. H.	1:25	1 g	+	±	0	+	+	±	Strongly positive,	3+
	A. E. B. H.	1:25	1 g	+	±	0	+	+	±	Strongly positive,	3+
	A. E. D. H.	1:30	1 g	+	±	0	+	+	±	Strongly positive,	3+
	A. E. G-P. H.	1:30	1 g	+	±	0	+	+	±	Strongly positive,	3+
	A. E. R. H.	1:25	1 g	+	±	0	+	+	±	Strongly positive,	3+
	A. E. S. H.	1:25	1 g	+	+	0	+	+	±	Moderately positive,	2+
53	A. E. H. H.	1:25	1 g	+	±	0	+	+	tr	Moderately positive,	2+
	A. E. B. H.	1:25	1 g	+	±	0	+	+	tr	Moderately positive,	2+
	A. E. D. H.	1:30	1 g	+	±	0	+	+	tr	Moderately positive,	2+
	A. E. G-P. H.	1:30	1 g	+	±	0	+	+	tr	Moderately positive,	2+
	A. E. R. P.	1:25	1 g	+	±	0	+	+	tr	Moderately positive,	2+
	A. E. S. H.	1:25	1 g	+	+	tr	+	+	tr	Negative,	—.
54	A. E. H. H.	1:25	1 g	+	tr	0	+	+	tr	Strongly positive,	3+
	A. E. B. H.	1:25	1 g	+	tr	0	+	+	tr	Strongly positive,	3+
	A. E. D. H.	1:30	1 g	+	tr	0	+	+	tr	Strongly positive,	3+
	A. E. G-P. H.	1:30	1 g	+	tr	0	+	+	tr	Strongly positive,	3+
	A. E. R. H.	1:25	1 g	+	tr	0	+	+	tr	Strongly positive,	3+
	A. E. S. H.	1:25	1 g	+	±	0	+	+	tr	Moderately positive,	2+

TABLE 1—Continued
COMPARISON OF ANTIGENS

Number of Serum	Kind of Antigen	Dilution of Antigen	Amboceptor per Tube, Unit	Readings*						Results	
				Antigen Tubes			Control Tubes				
				1	2	3	1'	2'	3'		
55	A. E. H. H.	1:25	1 g	+	0	0	+	+	±	Strongly positive,	5+.
	A. E. B. H.	1:25	1 g	±	0	0	+	+	±	Strongly positive,	6+.
	A. E. D. H.	1:30	1 g	+	0	0	+	+	±	Strongly positive,	5+.
	A. E. G-P. H.	1:30	1 g	+	0	0	+	+	±	Strongly positive,	5+.
	A. E. R. H.	1:25	1 g	+	0	0	+	+	±	Strongly positive,	5+.
	A. E. S. H.	1:25	1 g	+	tr	0	+	+	±	Strongly positive,	4+.
56	A. E. H. H.	1:25	1 g	+	tr	0	+	+	±	Strongly positive,	4+.
	A. E. B. H.	1:25	1 g	+	tr	0	+	+	±	Strongly positive,	4+.
	A. E. D. H.	1:30	1 g	+	tr	0	+	+	±	Strongly positive,	4+.
	A. E. G-P. H.	1:30	1 g	+	tr	0	+	+	±	Strongly positive,	4+.
	A. E. R. H.	1:25	1 g	+	tr	0	+	+	±	Strongly positive,	4+.
	A. E. S. H.	1:25	1 g	+	±	0	+	+	±	Strongly positive,	3+.
57	A. E. H. H.	1:25	1 g	±	0	0	+	+	tr	Strongly positive,	5+.
	A. E. B. H.	1:25	1 g	tr	0	0	+	+	tr	Strongly positive,	6+.
	A. E. D. H.	1:30	1 g	±	0	0	+	+	tr	Strongly positive,	5+.
	A. E. G-P. H.	1:30	1 g	±	0	0	+	+	tr	Strongly positive,	5+.
	A. E. R. H.	1:25	1 g	±	0	0	+	+	tr	Strongly positive,	5+.
	A. E. S. H.	1:25	1 g	+	0	0	+	+	tr	Strongly positive,	4+.
58	A. E. H. H.	1:25	1 g	+	±	0	+	+	±	Strongly positive,	3+.
	A. E. B. H.	1:25	1 g	+	0	0	+	+	±	Strongly positive,	5+.
	A. E. D. H.	1:30	1 g	+	tr	0	+	+	±	Strongly positive,	4+.
	A. E. G-P. H.	1:30	1 g	+	tr	0	+	+	±	Strongly positive,	4+.
	A. E. R. H.	1:25	1 g	+	0	0	+	+	±	Strongly positive,	5+.
	A. E. S. H.	1:25	1 g	+	±	0	+	+	±	Strongly positive,	3+.
59	A. E. H. H.	1:25	1 g	+	tr	0	+	+	±	Strongly positive,	4+.
	A. E. B. H.	1:25	1 g	+	0	0	+	+	±	Strongly positive,	5+.
	A. E. D. H.	1:30	1 g	+	tr	0	+	+	±	Strongly positive,	4+.
	A. E. G-P. H.	1:30	1 g	+	tr	0	+	+	±	Strongly positive,	4+.
	A. E. R. H.	1:25	1 g	+	tr	0	+	+	±	Strongly positive,	4+.
	A. E. S. H.	1:25	1 g	+	±	0	+	+	±	Strongly positive,	3+.
60	A. E. H. H.	1:25	1 g	±	0	0	+	+	±	Strongly positive,	6+.
	A. E. B. H.	1:25	1 g	0	0	0	+	+	±	Strongly positive,	10+.
	A. E. D. H.	1:30	1 g	tr	0	0	+	+	±	Strongly positive,	8+.
	A. E. G-P. H.	1:30	1 g	tr	0	0	+	+	±	Strongly positive,	8+.
	A. E. R. H.	1:25	1 g	tr	0	0	+	+	±	Strongly positive,	8+.
	A. E. S. H.	1:25	1 g	±	0	0	+	+	±	Strongly positive,	6+.
61	A. E. H. H.	1:25	1 g	+	0	0	+	+	±	Strongly positive,	5+.
	A. E. B. H.	1:25	1 g	+	0	0	+	+	±	Strongly positive,	5+.
	A. E. D. H.	1:30	1 g	+	0	0	+	+	±	Strongly positive,	5+.
	A. E. G-P. H.	1:30	1 g	+	0	0	+	+	±	Strongly positive,	5+.
	A. E. R. H.	1:25	1 g	+	0	0	+	+	±	Strongly positive,	5+.
	A. E. S. H.	1:25	1 g	+	tr	0	+	+	±	Strongly positive,	4+.
62	A. E. H. H.	1:25	1 g	+	tr	0	+	+	±	Strongly positive,	4+.
	A. E. B. H.	1:25	1 g	+	tr	0	+	+	±	Strongly positive,	4+.
	A. E. D. H.	1:30	1 g	+	±	0	+	+	±	Strongly positive,	3+.
	A. E. G-P. H.	1:30	1 g	+	±	0	+	+	±	Strongly positive,	3+.
	A. E. R. H.	1:25	1 g	+	0	0	+	+	±	Strongly positive,	5+.
	A. E. S. H.	1:25	1 g	+	±	0	+	+	±	Strongly positive,	3+.
63	A. E. H. H.	1:25	1 g	+	tr	0	+	+	±	Strongly positive,	4+.
	A. E. B. H.	1:25	1 g	+	tr	0	+	+	±	Strongly positive,	4+.
	A. E. D. H.	1:30	1 g	+	±	0	+	+	±	Strongly positive,	3+.
	A. E. G-P. H.	1:30	1 g	+	±	0	+	+	±	Strongly positive,	3+.
	A. E. R. H.	1:25	1 g	+	0	0	+	+	±	Strongly positive,	5+.
	A. E. S. H.	1:25	1 g	+	+	tr	+	+	±	Weakly positive,	1+.

17 (34%). All the serums which gave positive results with the alcoholic extract of dog heart also gave positive results with the alcoholic extract of guinea-pig heart; the results were usually stronger positive with the alcoholic extract of guinea-pig heart than with the alcoholic extract of dog heart, never the reverse.

With the serums from persons known to be syphilitic the alcoholic extract of beef heart and the alcoholic extract of rabbit heart frequently gave stronger positive results than did the alcoholic extract of human heart. With the alcoholic extract of dog heart and of guinea-pig heart the results corresponded to those obtained with human antigen while with the alcoholic extract of sheep heart the results were somewhat weaker.

TABLE 2
ANTIGEN ADDED AFTER AND BEFORE THE COMPLEMENT

Number of Serum	Kind of Antigen	Antigen Added A=After B=Before	Amboceptor per Tube, Unit	Readings*						Results
				Antigen Tubes			Control Tubes			
				1	2	3	1'	2'	3'	
64	A. E. H. H.	A	1 g	+	0	0	+	+	tr	Strongly positive, 4+.
	A. E. H. H.	B	1 g	+	0	0	+	+	tr	Strongly positive, 4+.
65	A. E. H. H.	A	1 g	+	tr	0	+	+	±	Strongly positive, 4+.
	A. E. H. H.	B	1 g	+	tr	0	+	+	±	Strongly positive, 4+.
66	A. E. H. H.	A	1 g	+	±	0	+	+	±	Strongly positive, 3+.
	A. E. H. H.	B	1 g	+	±	0	+	+	±	Strongly positive, 3+.
67	A. E. H. H.	A	1 g	±	0	0	+	+	±	Strongly positive, 6+.
	A. E. H. H.	B	1 g	+	0	0	+	+	±	Strongly positive, 5+.
68	A. E. H. H.	A	1 g	+	tr	0	+	+	±	Strongly positive, 4+.
	A. E. H. H.	B	1 g	+	tr	0	+	+	±	Strongly positive, 4+.
69	A. E. H. H.	A	1 g	+	tr	0	+	+	±	Strongly positive, 4+.
	A. E. H. H.	B	1 g	+	tr	0	+	+	±	Strongly positive, 4+.
70	A. E. H. H.	A	1 g	+	±	0	+	+	±	Strongly positive, 3+.
	A. E. H. H.	B	1 g	+	±	0	+	+	±	Strongly positive, 3+.

* Explanation: + means complete hemolysis; ±, hemolysis between 50% and 100%; tr (trace), hemolysis less than 50%; 0, no hemolysis.

Seven human serums, 64-70, inclusive, were used to determine whether the order in which human serum, complement serum and antigen are mixed is of importance. Two sets of test tubes were taken for each serum, and each tube received the serum to be tested. The tubes of the first set received complement, and the antigen tubes in the second set received the antigen. Fifteen minutes later the antigen tubes of the first set received the antigen, all tubes in the second set received complement, and all control tubes received salt solution. After human serum, complement and antigen had been mixed all tubes were placed in the refrigerator at about 10 C. for 5 hours.

The results obtained with Serums 64-70, inclusive, are shown in Table 2. With 6 of the 7, Serums 64, 65, 66, 68, 69 and 70, identical results were obtained by the 2 methods. Serum 67 gave 6 + when the antigen was added 15 minutes later than the complement and 5 + when the antigen was added 15 minutes before the complement.

With Serums 71-115, inclusive, different dilutions of antigen were compared with a dilution of 1:25. The antigen was diluted so as to get a turbid solution, complement was a mixture of serum from 3 guinea-pigs and was used on the 1st and 2nd days after the bleeding. First incubation was in the refrigerator at about 10 C. for 5 hours; second incubation was in the incubator at about 37 C. for 1 hour, and the results were read from 1-2 hours after the tubes had been removed from the incubator.

Table 3 shows the results obtained with different dilutions of antigen. The results were somewhat irregular. Dilutions 1:35, 1:45, 1:50 and 1:75 gave results identical with those obtained with a dilution of 1:25. Dilutions of 1:100, 1:200 and 1:300 gave irregular results, some were identical with those given by a dilution of 1:25, some were stronger positive and others were weaker positive. Dilutions higher than 1:300 usually gave weaker positive results than did a dilution of 1:25. The power to bind complement disappeared gradually, about 20% was still bound by a dilution of 1:1000.

SUMMARY

With serums from 50 clinically nonsyphilitic persons alcoholic extract of human heart, alcoholic extract of beef heart and alcoholic extract of rabbit heart gave negative results. Alcoholic extract of dog heart gave positive results with 16, or 32%, and alcoholic extract of guinea-pig heart gave positive results with 17, or 34%, of these serums from clinically nonsyphilitic persons. As no other signs or symptoms of syphilis could be discovered these were considered false positive results, and heart of dog and of guinea-pig are considered unsuitable for the preparation of so-called antigen to be used in the Wassermann reaction. Whenever the alcoholic extract of dog heart gave a positive result the alcoholic extract of guinea-pig heart also gave a positive result; the latter usually reacted somewhat stronger than did the former.

With serum from known syphilitics the alcoholic extract of beef heart and the alcoholic extract of rabbit heart frequently gave stronger positive results than did the alcoholic extract of human heart. The results obtained with alcoholic extracts of dog heart and guinea-pig

TABLE 3
DIFFERENT DILUTIONS OF ANTIGEN COMPARED

Number of Serum	Kind of Antigen	Dilution of Antigen	Antibody per Tube, Unit	Readings*						Results
				Antigen Tubes			Control Tubes			
				1	2	3	1'	2'	3	
71	A. E. H. H.	1:25	1 g	+	0	0	+	+	±	Strongly positive, 5+.
	A. E. H. H.	1:35	1 g	+	0	0	+	+	±	Strongly positive, 5+.
	A. E. H. H.	1:45	1 g	+	0	0	+	+	±	Strongly positive, 5+.
72	A. E. H. H.	1:25	1 g	±	0	0	+	+	±	Strongly positive, 6+.
	A. E. H. H.	1:35	1 g	±	0	0	+	+	±	Strongly positive, 6+.
	A. E. H. H.	1:45	1 g	±	0	0	+	+	±	Strongly positive, 6+.
73	A. E. H. H.	1:25	1 g	+	0	0	+	+	±	Strongly positive, 5+.
	A. E. H. H.	1:35	1 g	+	0	0	+	+	±	Strongly positive, 5+.
	A. E. H. H.	1:45	1 g	+	0	0	+	+	±	Strongly positive, 5+.
74	A. E. H. H.	1:25	1 g	+	0	0	+	+	±	Strongly positive, 5+.
	A. E. H. H.	1:35	1 g	+	0	0	+	+	±	Strongly positive, 5+.
	A. E. H. H.	1:45	1 g	+	0	0	+	+	±	Strongly positive, 5+.
75	A. E. H. H.	1:25	1 g	+	0	0	+	+	±	Strongly positive, 5+.
	A. E. H. H.	1:35	1 g	+	0	0	+	+	±	Strongly positive, 5+.
	A. E. H. H.	1:45	1 g	+	0	0	+	+	±	Strongly positive, 5+.
76	A. E. H. H.	1:25	1 g	+	tr	0	+	+	±	Strongly positive, 4+.
	A. E. H. H.	1:50	1 g	+	tr	0	+	+	±	Strongly positive, 4+.
	A. E. H. H.	1:75	1 g	+	tr	0	+	+	±	Strongly positive, 4+.
77	A. E. H. H.	1:25	1 g	+	+	0	+	+	±	Moderately positive, 2+.
	A. E. H. H.	1:50	1 g	+	±	0	+	+	±	Strongly positive, 3+.
	A. E. H. H.	1:75	1 g	+	±	0	+	+	±	Strongly positive, 3+.
78	A. E. H. H.	1:25	1 g	+	0	0	+	+	±	Strongly positive, 5+.
	A. E. H. H.	1:50	1 g	+	0	0	+	+	±	Strongly positive, 5+.
	A. E. H. H.	1:75	1 g	+	0	0	+	+	±	Strongly positive, 5+.
79	A. E. H. H.	1:25	1 g	+	tr	0	+	+	tr	Strongly positive, 3+.
	A. E. H. H.	1:50	1 g	+	tr	0	+	+	tr	Strongly positive, 3+.
	A. E. H. H.	1:75	1 g	+	tr	0	+	+	tr	Strongly positive, 3+.
80	A. E. H. H.	1:25	1 g	+	0	0	+	+	tr	Strongly positive, 4+.
	A. E. H. H.	1:50	1 g	+	0	0	+	+	tr	Strongly positive, 4+.
	A. E. H. H.	1:75	1 g	+	0	0	+	+	tr	Strongly positive, 4+.
81	A. E. H. H.	1:25	1 g	+	±	0	+	+	±	Strongly positive, 3+.
	A. E. H. H.	1:100	1 g	+	±	0	+	+	±	Strongly positive, 3+.
	A. E. H. H.	1:200	1 g	+	±	0	+	+	±	Strongly positive, 3+.
82	A. E. H. H.	1:25	1 g	+	+	0	+	+	±	Moderately positive, 2+.
	A. E. H. H.	1:100	1 g	+	±	0	+	+	±	Strongly positive, 3+.
	A. E. H. H.	1:200	1 g	+	±	0	+	+	±	Strongly positive, 3+.
83	A. E. H. H.	1:25	1 g	+	+	0	+	+	±	Moderately positive, 2+.
	A. E. H. H.	1:100	1 g	+	±	0	+	+	±	Strongly positive, 3+.
	A. E. H. H.	1:200	1 g	+	±	0	+	+	±	Strongly positive, 3+.
84	A. E. H. H.	1:25	1 g	+	tr	0	+	+	±	Strongly positive, 4+.
	A. E. H. H.	1:100	1 g	+	0	0	+	+	±	Strongly positive, 5+.
	A. E. H. H.	1:200	1 g	+	tr	0	+	+	±	Strongly positive, 4+.
85	A. E. H. H.	1:25	1 g	+	±	0	+	+	±	Strongly positive, 3+.
	A. E. H. H.	1:100	1 g	+	tr	0	+	+	±	Strongly positive, 4+.
	A. E. H. H.	1:200	1 g	+	tr	0	+	+	±	Strongly positive, 4+.
86	A. E. H. H.	1:25	1 g	+	±	0	+	+	±	Strongly positive, 3+.
	A. E. H. H.	1:300	1 g	+	±	0	+	+	±	Strongly positive, 3+.
	A. E. H. H.	1:400	1 g	+	+	0	+	+	±	Moderately positive, 2+.

TABLE 3—Continued
DIFFERENT DILUTIONS OF ANTIGEN COMPARED

Number of Serum	Kind of Antigen	Dilution of Antigen	Antiboeceptor per Tube, Unit	Readings*						Results
				Antigen Tubes			Control Tubes			
				1	2	3	1'	2'	3	
87	A. E. H. H.	1:25	1 g	+	+	0	+	+	±	Moderately positive, 2+. Strongly positive, 3+. Moderately positive, 2+.
	A. E. H. H.	1:300	1 g	+	±	0	+	+	±	
	A. E. H. H.	1:400	1 g	+	+	0	+	+	±	
88	A. E. H. H.	1:25	1 g	+	+	0	+	+	±	Moderately positive, 2+. Moderately positive, 2+. Weakly positive, 1+.
	A. E. H. H.	1:300	1 g	+	+	0	+	+	±	
	A. E. H. H.	1:400	1 g	+	+	tr	+	+	±	
89	A. E. H. H.	1:25	1 g	+	0	0	+	+	±	Strongly positive, 5+. Strongly positive, 5+. Strongly positive, 3+.
	A. E. H. H.	1:300	1 g	+	0	0	+	+	±	
	A. E. H. H.	1:400	1 g	+	±	0	+	+	±	
90	A. E. H. H.	1:25	1 g	+	±	0	+	+	±	Strongly positive, 3+. Strongly positive, 3+. Moderately positive, 2+.
	A. E. H. H.	1:300	1 g	+	±	0	+	+	±	
	A. E. H. H.	1:400	1 g	+	+	0	+	+	±	
91	A. E. H. H.	1:25	1 g	+	tr	0	+	+	±	Strongly positive, 4+. Moderately positive, 2+. Moderately positive, 2+.
	A. E. H. H.	1:500	1 g	+	+	0	+	+	±	
	A. E. H. H.	1:600	1 g	+	+	0	+	+	±	
92	A. E. H. H.	1:25	1 g	+	±	0	+	+	±	Strongly positive, 3+. Moderately positive, 2+. Moderately positive, 2+.
	A. E. H. H.	1:500	1 g	+	+	0	+	+	±	
	A. E. H. H.	1:600	1 g	+	+	0	+	+	±	
93	A. E. H. H.	1:25	1 g	+	±	0	+	+	±	Strongly positive, 3+. Moderately positive, 2+. Moderately positive, 2+.
	A. E. H. H.	1:500	1 g	+	+	0	+	+	±	
	A. E. H. H.	1:600	1 g	+	+	0	+	+	±	
94	A. E. H. H.	1:25	1 g	+	tr	0	+	+	±	Strongly positive, 4+. Weakly positive, 1+. Weakly positive, 1+.
	A. E. H. H.	1:500	1 g	+	+	tr	+	+	±	
	A. E. H. H.	1:600	1 g	+	+	tr	+	+	±	
95	A. E. H. H.	1:25	1 g	+	tr	0	+	+	±	Strongly positive, 4+. Weakly positive, 1+. Weakly positive, 1+.
	A. E. H. H.	1:500	1 g	+	+	tr	+	+	±	
	A. E. H. H.	1:600	1 g	+	+	tr	+	+	±	
96	A. E. H. H.	1:25	1 g	+	0	0	+	+	±	Strongly positive, 5+. Faintly positive, ±. Faintly positive, ±.
	A. E. H. H.	1:800	1 g	+	+	±?	+	+	±	
	A. E. H. H.	1:1,000	1 g	+	+	±?	+	+	±	
97	A. E. H. H.	1:25	1 g	+	tr	0	+	+	±	Strongly positive, 4+. Weakly positive, 1+. Faintly positive, ±.
	A. E. H. H.	1:800	1 g	+	+	tr	+	+	±	
	A. E. H. H.	1:1,000	1 g	+	+	±?	+	+	±	
98	A. E. H. H.	1:25	1 g	+	0	0	+	+	±	Strongly positive, 5+. Moderately positive, 2+. Weakly positive, 1+.
	A. E. H. H.	1:800	1 g	+	+	0	+	+	±	
	A. E. H. H.	1:1,000	1 g	+	+	tr	+	+	±	
99	A. E. H. H.	1:25	1 g	+	±	0	+	+	±	Strongly positive, 3+. Weakly positive, 1+. Faintly positive, ±.
	A. E. H. H.	1:800	1 g	+	+	tr	+	+	±	
	A. E. H. H.	1:1,000	1 g	+	+	±?	+	+	±	
100	A. E. H. H.	1:25	1 g	±	0	0	+	+	±	Strongly positive, 6+. Strongly positive, 3+. Moderately positive, 2+.
	A. E. H. H.	1:800	1 g	+	±	0	+	+	±	
	A. E. H. H.	1:1,000	1 g	+	+	0	+	+	±	
101	A. E. H. H.	1:25	1 g	+	+	tr	+	+	tr	Negative, —.
	A. E. H. H.	1:100	1 g	+	+	tr	+	+	tr	Negative, —.
	A. E. H. H.	1:200	1 g	+	+	tr	+	+	tr	Negative, —.
	A. E. H. H.	1:400	1 g	+	+	tr	+	+	tr	Negative, —.
	A. E. H. H.	1:600	1 g	+	+	tr	+	+	tr	Negative, —.

TABLE 3—Continued
DIFFERENT DILUTIONS OF ANTIGEN COMPARED

Number of Serum	Kind of Antigen	Dilution of Antigen	Amboceptor per Tube, Unit	Readings*						Results	
				Antigen Tubes			Control Tubes				
				1	2	3	1'	2'	3		
102	A. E. H. H.	1:25	1 g	+	+	tr	+	+	tr	Negative,	—.
	A. E. H. H.	1:100	1 g	+	+	tr	+	+	tr	Negative,	—.
	A. E. H. H.	1:200	1 g	+	+	tr	+	+	tr	Negative,	—.
	A. E. H. H.	1:400	1 g	+	+	tr	+	+	tr	Negative,	—.
	A. E. H. H.	1:600	1 g	+	+	tr	+	+	tr	Negative,	—.
103	A. E. H. H.	1:25	1 g	+	+	±	+	+	±	Negative,	—.
	A. E. H. H.	1:100	1 g	+	+	±	+	+	±	Negative,	—.
	A. E. H. H.	1:200	1 g	+	+	±	+	+	±	Negative,	—.
	A. E. H. H.	1:400	1 g	+	+	±	+	+	±	Negative,	—.
	A. E. H. H.	1:600	1 g	+	+	±	+	+	±	Negative,	—.
104	A. E. H. H.	1:25	1 g	+	tr	0	+	+	±	Strongly positive,	4+.
	A. E. H. H.	1:100	1 g	+	tr	0	+	+	±	Strongly positive,	4+.
	A. E. H. H.	1:200	1 g	+	±	0	+	+	±	Strongly positive,	3+.
	A. E. H. H.	1:400	1 g	+	+	0	+	+	±	Moderately positive,	2+.
	A. E. H. H.	1:600	1 g	+	+	0	+	+	±	Moderately positive,	2+.
105	A. E. H. H.	1:25	1 g	+	tr	0	+	+	±	Strongly positive,	4+.
	A. E. H. H.	1:100	1 g	+	0	0	+	+	±	Strongly positive,	5+.
	A. E. H. H.	1:200	1 g	+	0	0	+	+	±	Strongly positive,	5+.
	A. E. H. H.	1:400	1 g	+	±	0	+	+	±	Strongly positive,	3+.
	A. E. H. H.	1:600	1 g	+	+	0	+	+	±	Moderately positive,	2+.
106	A. E. H. H.	1:25	1 g	+	tr	0	+	+	±	Strongly positive,	4+.
	A. E. H. H.	1:100	1 g	+	tr	0	+	+	±	Strongly positive,	4+.
	A. E. H. H.	1:200	1 g	+	tr	0	+	+	±	Strongly positive,	4+.
	A. E. H. H.	1:400	1 g	+	±	0	+	+	±	Strongly positive,	3+.
	A. E. H. H.	1:600	1 g	+	±	0	+	+	±	Strongly positive,	3+.
107	A. E. H. H.	1:25	1 g	+	±	0	+	+	±	Strongly positive,	3+.
	A. E. H. H.	1:100	1 g	+	+	0	+	+	±	Moderately positive,	2+.
	A. E. H. H.	1:200	1 g	+	+	0	+	+	±	Moderately positive,	2+.
	A. E. H. H.	1:400	1 g	+	+	tr	+	+	±	Weakly positive,	1+.
	A. E. H. H.	1:600	1 g	+	+	±?	+	+	±	Faintly positive,	±.
108	A. E. H. H.	1:25	1 g	+	+	0	+	+	±	Moderately positive,	2+.
	A. E. H. H.	1:100	1 g	+	±	0	+	+	±	Strongly positive,	3+.
	A. E. H. H.	1:200	1 g	+	±	0	+	+	±	Strongly positive,	3+.
	A. E. H. H.	1:400	1 g	+	+	tr	+	+	±	Weakly positive,	1+.
	A. E. H. H.	1:600	1 g	+	+	tr	+	+	±	Weakly positive,	1+.
109	A. E. H. H.	1:25	1 g	+	tr	0	+	+	±	Strongly positive,	4+.
	A. E. H. H.	1:100	1 g	+	tr	0	+	+	±	Strongly positive,	4+.
	A. E. H. H.	1:200	1 g	+	±	0	+	+	±	Strongly positive,	3+.
	A. E. H. H.	1:400	1 g	+	+	0	+	+	±	Moderately positive,	2+.
	A. E. H. H.	1:600	1 g	+	+	tr	+	+	±	Weakly positive,	1+.
110	A. E. H. H.	1:25	1 g	+	0	0	+	+	±	Strongly positive,	5+.
	A. E. H. H.	1:100	1 g	+	tr	0	+	+	±	Strongly positive,	4+.
	A. E. H. H.	1:200	1 g	+	±	0	+	+	±	Strongly positive,	3+.
	A. E. H. H.	1:400	1 g	+	+	0	+	+	±	Moderately positive,	2+.
	A. E. H. H.	1:600	1 g	+	+	tr	+	+	±	Weakly positive,	1+.
111	A. E. H. H.	1:25	1 g	+	tr	0	+	+	±	Strongly positive,	4+.
	A. E. H. H.	1:100	1 g	+	±	0	+	+	±	Strongly positive,	3+.
	A. E. H. H.	1:200	1 g	+	+	0	+	+	±	Moderately positive,	2+.
	A. E. H. H.	1:400	1 g	+	+	tr	+	+	±	Weakly positive,	1+.
	A. E. H. H.	1:600	1 g	+	+	±?	+	+	±	Faintly positive,	±.

TABLE 3—Continued
DIFFERENT DILUTIONS OF ANTIGEN COMPARED

Number of Serum	Kind of Antigen	Dilution of Antigen	Amboceptor per Tube, Unit	Readings*						Results
				Antigen Tubes			Control Tubes			
				1	2	3	1'	2'	3'	
112	A. E. H. H.	1:25	1 g	+	0	0	+	+	±	Strongly positive, 5+.
	A. E. H. H.	1:100	1 g	+	tr	0	+	+	±	Strongly positive, 4+.
	A. E. H. H.	1:200	1 g	+	±	0	+	+	±	Strongly positive, 3+.
	A. E. H. H.	1:400	1 g	+	+	0	+	+	±	Moderately positive, 2+.
	A. E. H. H.	1:600	1 g	+	+	tr	+	+	±	Weakly positive, 1+.
113	A. E. H. H.	1:25	1 g	+	+	0	+	+	±	Moderately positive, 2+.
	A. E. H. H.	1:100	1 g	+	±	0	+	+	±	Strongly positive, 3+.
	A. E. H. H.	1:200	1 g	+	±	0	+	+	±	Strongly positive, 3+.
	A. E. H. H.	1:400	1 g	+	+	0	+	+	±	Moderately positive, 2+.
	A. E. H. H.	1:600	1 g	+	+	tr	+	+	±	Weakly positive, 1+.
114	A. E. H. H.	1:25	1 g	+	±	0	+	+	±	Strongly positive, 3+.
	A. E. H. H.	1:100	1 g	+	±	0	+	+	±	Strongly positive, 3+.
	A. E. H. H.	1:200	1 g	+	±	0	+	+	±	Strongly positive, 3+.
	A. E. H. H.	1:400	1 g	+	+	0	+	+	±	Moderately positive, 2+.
	A. E. H. H.	1:600	1 g	+	+	0	+	+	±	Moderately positive, 2+.
115	A. E. H. H.	1:25	1 g	+	+	0	+	+	±	Moderately positive, 2+.
	A. E. H. H.	1:100	1 g	+	±	0	+	+	±	Strongly positive, 3+.
	A. E. H. H.	1:200	1 g	+	±	0	+	+	±	Strongly positive, 3+.
	A. E. H. H.	1:400	1 g	+	+	0	+	+	±	Moderately positive, 2+.
	A. E. H. H.	1:600	1 g	+	+	tr	+	+	±	Weakly positive, 1+.

* Explanation: + means complete hemolysis; ±, hemolysis between 50% and 100%; tr (trace), hemolysis less than 50%; 0, no hemolysis.

heart corresponded to those obtained with alcoholic extract of human heart, while the alcoholic extract of sheep heart reacted weaker.

When antigen was added 15 minutes after the complement the same results were obtained as when antigen was added 15 minutes before the complement.

Antigen dilutions of 1:35, 1:45, 1:50 and 1:75 gave results that were identical with those given by a dilution of 1:25. Dilutions of 1:100, 1:200 and 1:300 gave irregular results, sometimes stronger, sometimes identical with and other times weaker than those given by a dilution of 1:25. Dilutions higher than 1:300 usually gave weaker positive results than did the dilution of 1:25.

DROPLET INFECTION AND ITS PREVENTION BY THE FACE MASK

GEORGE H. WEAVER

From the John McCormick Institute for Infectious Diseases, Chicago.

In recent years the spread of contagious diseases has been combated largely by measures calculated to limit the more or less direct passage or carriage of infectious materials from the sick to others. The term contact infection has often been employed to designate all such instances of direct passage or carriage, although actual contact did not always occur. Aerial transfer of infectious materials has been applied to a wide distribution of disease agents through air at considerable distances, and especially to dissemination through dust. This form of transfer has been shown to play so small a part in the spread of contagious diseases as to be practically negligible. The part played in the transfer of infections by mouth droplets driven out in forced expiratory efforts has not usually received sufficient attention. The tendency of those who have insisted on the almost exclusive rôle of contact infection in the spread of contagious diseases has been to include droplet infection among the forms of contact infection, but to assign it a minor part. The factor of distance which is a most important one has been largely ignored.

Recent experiences have served to emphasize the ease with which infections may be transferred through mouth droplets when people are brought into intimate association in military establishments. The danger of transfer in this way of secondary infecting organisms which cause most complications in cases of contagious diseases has long been appreciated by physicians who have dealt with these diseases in institutions, and they have insisted on the isolation of individuals who have active secondary infections from others who have the uncomplicated disease. Secondary infections are transferred in the same manner as the primary disease in most instances. Our recent army experiences have emphasized the fact that carriers and droplet infections are two factors which must receive a large share of attention in the management of contagious diseases.

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Intimate contact of individuals is essential in order that droplet infection may occur, and this applies equally to single persons and to larger numbers in camps, crowded cars or public gatherings within doors.

That crossed infections among patients with contagious diseases can be almost eliminated if the individuals are separated sufficiently to eliminate droplet infection and measures are taken to avoid direct carriage of infectious materials was first practically appreciated by French physicians and incorporated by them in practice in hospitals. Similar methods were soon adopted by British isolation hospitals and in this country aseptic methods in the management of contagious diseases has come into general use, largely through the consistent advocacy of Chapin and his pupils.

The droplets of mouth spray consist largely of saliva, and they are carriers of infectious materials in proportion as such are present in the mouth. Tubercle bacilli have been found in the saliva and on the tongue in a considerable proportion of cases of pulmonary tuberculosis. Diphtheria bacilli have been found on articles contaminated by saliva from persons with diphtheria. Teague¹ found diphtheria bacilli in the saliva in 77% of cases in which tonsillar cultures were positive. We have examined cultures from the tip and sides of the tongue of individuals with diphtheria and have frequently found the bacillus in this location. In any cases in which pathogenic organisms are present in pharynx, nasopharynx and in sputum from the deeper respiratory passages, it is likely that the mouth will be more or less contaminated by them and that they will be in the saliva. Many pathogenic bacteria have been found in mouth spray. Tubercle bacilli in mouth spray have been demonstrated by numerous observers, and guinea-pigs have been infected by exposure to the mouth spray of tuberculous patients.² In a recent study of tubercle bacilli isolated from sputum, Corper³ says that "there is only one conclusion to be drawn from these findings as viewed from a practical standpoint, and that is that the tubercle bacilli discharged by droplet or by expectoration from open cases of pulmonary tuberculosis are a danger to mankind on direct transmission at least."

Teague¹ found that over one-half of diphtheria patients emitted diphtheria bacilli in talking and coughing, the plates being exposed for

¹ Jour. Infect. Dis., 1912, 12, p. 398.

² Heymann: Ztschr. f. Hyg. u. Infektionskrankh., 30, p. 139.

³ Tr. Chicago Path. Soc., 1918, 10, p. 227.

a very short time. Hamilton,⁴ in 1905, found that scarlet fever patients frequently threw out streptococci in invisible sputum. We have repeated her experiments and have found that hemolytic streptococci are often emitted in considerable numbers from the mouth of scarlet fever patients during coughing.

The occurrence of infection after exposure to mouth spray depends on several factors, especially the immunity of the individual and the number of bacteria taken in. The latter factor will vary much, and single bacteria carried to a distance would be relatively less dangerous than clumps of bacteria in heavier droplets which settle from the air before passing far from the patient. Immune individuals may, nevertheless, become carriers without exhibiting any evidence of infection. The distance to which mouth droplets are carried in the air depends principally on the force with which they are driven. Small droplets may pass some distance, especially when carried by currents of air. The observations of Doust and Lyon⁵ show that the distance to which droplets are projected in quiet air is much greater than usually supposed; the "danger zone about a coughing patient has at least a 10 foot radius." Our experience would indicate that relatively few bacteria pass more than a few feet from the patient in ordinary coughing in the absence of currents of air.

Those who have studied the bacterial content of mouth spray have remarked on the great variation in the number of colonies developing after coughing toward exposed plates. This variation is partly explained by the manner of coughing. Coughing efforts which force the expired air through a relatively narrow opening produce many more colonies than do those made with the lips more widely separated. Forcible expiratory efforts carried out with the lips only slightly opened produce the most abundant droplet spray. The relative number of colonies developing after various expiratory efforts are shown in Table 1.

When the Durand Hospital of this institute was opened, rigid aseptic methods were adopted, and the nurses were specially instructed in measures calculated to protect them from infections. From March 12, 1913, to Nov. 1, 1914, nine out of 69 nurses, or 13%, acquired clinical diphtheria. From this time on, all nurses giving a positive Schick test were immunized with diphtheria antitoxin. This practically

⁴ Jour. Am. Med. Assn., 1905, 44, p. 1108.

⁵ Jour. Am. Med. Assn., 1918, 71, p. 1216.

eliminated active diphtheria, but from Nov. 1, 1914, to June 1, 1916, weekly throat cultures disclosed 10 diphtheria bacillus carriers among 43 nurses, or 23.25%. Up to June 1, 1916, nine cases of scarlet fever occurred among 112 nurses on duty, or in 8%.

TABLE 1
COLONIES AFTER VARIOUS EXPIRATORY EFFORTS

Showing number of colonies of *Streptococcus viridans* developing on blood-agar plates exposed at a distance of 1 foot during various expiratory efforts. The figures are the average of several experiments made with the same person as was employed in the experiments shown in the following tables.

Expiratory Efforts Employed	Number of Colonies
Talking (15 seconds)	1
Coughing with lips widely open (twice).....	1
Whistling (15 seconds)	2
Whispering faintly (15 seconds)	4
Whispering loudly (15 seconds).....	5
Blowing (twice)	50
Stuttering in a whisper (15 seconds)	55
Hawking (once)	100
Stuttering loudly (15 seconds)	100
Coughing with lips slightly parted (twice)	200
Sneezing (once)	300
Lips forced slightly apart with a puff (twice)	670

Being unable to explain so many instances of infection through faulty technic, an effort was made to eliminate a possible factor of danger which had previously been largely ignored, namely, infection through mouth spray. Since June 1, 1916,⁶ gauze masks have been used by the nurses, and up to Oct. 1, 1918, 6 diphtheria bacillus carriers have been detected among 73 nurses, or in 5.2%. No case of scarlet fever has occurred since masks have been worn. The nurses are instructed to change the mask as soon as it has been known to be grossly contaminated and never to put the hands to the mask to adjust it, etc., until they have been thoroughly washed.

Early in 1918 bacteriologic tests showed that the masks we were using did not remove all the bacteria thrown out in mouth spray. The masks consisted of 2 layers of gauze, 28 by 24 mesh, but as they were worn but once before washing and resterilizing, shrinkage soon made the opening in the gauze much closer than they were in the new masks. Studies were instituted, to learn how the masks could be made most efficient.

It was assumed that the power of various gauzes to filter moist spray from air would increase with closeness of mesh and with the number of layers employed. In the first tests a spray of carbolfuchsin

⁶ Our experiences up to Dec. 1, 1917, were reported in January, 1918, in the Jour. Am. Med. Assn., 1918, 70, p. 76.

was employed, the dye being susceptible of fairly accurate measurements.

A piece of cardboard 20 inches square was placed vertically on a table and an opening 4 inches square cut in it, the bottom of the opening being 4 inches from the table, and the sides equidistant from the sides of the cardboard. Back of the cardboard and opposite the opening uncovered petri dishes were placed vertically on a rack, the open side of the dish toward the opening. Toward the opening in the cardboard, with and without the interposition of gauze over the opening, a spray of carbolfuchsin was thrown by a hand atomizer. Two compressions of the bulb were used in each test and care was taken to make the compressions uniform in force. The amount of fuchsin lodging on the bottom of the dishes was determined by adding to each dish 5 cc of alcohol and pouring the alcohol with the dissolved dye into test tubes with a lumen of 1 cm. The tubes were then compared with similar tubes containing definite amounts of fuchsin dissolved in 5 cc of alcohol. In preparing the standard tubes the fuchsin lodging on a dish 6 inches from the spray with no gauze interposed was dissolved in 5 cc of alcohol and taken as 100%. The other units were made by diluting the 100% solution with alcohol. In dilutions of less than 0.1% color could not be detected.

The results of these tests are shown in Table 2.

On dishes at a distance of 4 feet from the spray the fuchsin was barely visible when dissolved in 5 cc of alcohol. The percentage of fuchsin lodging on the dishes becomes progressively less as the distance from the spray increases. The percentage of fuchsin passing through the gauze becomes less as the mesh of the gauze becomes closer and as the number of layers of gauze is multiplied.

Experiments were next made to determine how a spray of bacterial suspension would behave under conditions similar to those employed in testing the fuchsin solution. For these tests a suspension of *B. prodigiosus* in NaCl solution, 1 loop to 50 cc was employed. The tests were made as in the former case, except that the petri dishes contained nutrient agar. The dishes after exposure were incubated and colonies counted. The results are shown in Table 3.

It will be noted that the number of colonies became progressively less as the distance from the plates increased and also as the mesh of the gauze became finer and as the number of layers of gauze increased. It is interesting to note that at a distance of 3-5 feet from the spray the proportion of the bacteria reaching that point which passed through the gauze barriers was greater than at shorter or greater distances. This is probably to be explained by the more rapid precipitation of the larger particles as regards the nearer distances and by the failing force at the greater distances.

TABLE 3

B. PRODIGIOSUS EXPERIMENTS

Showing colonies developing on plates when suspension in NaCl solution of *Bacillus prodigiosus* is sprayed through gauze, placed 3 inches from plates, using 2 compressions of bulb.

The upper figures in the squares represent the number of colonies.
The lower figures in the squares represent the percentage of colonies as compared with the unobstructed plate at the same distance.

Distance from Spray to Plate	No Gauze	Mesh of Gauze											
		20 × 14			24 × 20			28 × 24			32 × 28		
		Layers			Layers			Layers			Layers		
		1	2	4	1	2	4	1	2	4	1	2	4
6 inches.....	40,000	40,000 100	40,000 100	20,000 50	40,000 100	40,000 100	1,500 3.75	6,000 15	2,000 5	1,200 3	6,000 15	1,200 3	60 0.15
1 foot.....	20,000	20,000 100	15,000 75	10,000 50	20,000 100	15,000 75	500 2.5	1,200 6	600 3	150 0.75	1,500 7.5	200 1	50 0.25
2 feet.....	6,000	4,000 66	1,000 16.6	500 8.3	900 15	1,200 20	300 5	300 5	200 3.3	50 0.83	300 5	200 3.3	60 1.0
3 feet.....	1,200	800 66.6	500 41.6	300 25	500 41.6	600 50	200 16	100 8	80 6.6	50 4.1	300 25	80 6.6	70 5.8
4 feet.....	400	400 100	300 75	200 50	400 100	200 50	150 37.5	90 22.5	20 5	40 10	90 22.5	50 12.5	40 10
5 feet.....	300	300 100	100 33	70 23.3	80 26.6	150 50	80 26.6	80 26.6	24 8	40 13.3	90 30	40 13.3	1 0.3
6 feet.....	250	150 60	80 32	20 8	40 16	50 20	13 5.2	9 3.6	10 4	20 8	80 32	20 5	1 0.4
7 feet.....	200	150 75	100 50	20 10	15 7.5	20 10	6 3	1 0.5	2 1.0	8 4.0	40 20	15 7.5	3 1.5
8 feet.....	80	80 100	30 37.5	20 25	7 8.75	12 15	3 3.75	0 0	0 0	0 0	25 31	0 0	0 0

These results demonstrate that gauze will remove bacteria from the air when carried in a moist spray. The efficiency of the gauze as a filter is in direct ratio to the fineness of the mesh and the number of layers used.

It was now desirable to determine the efficiency of gauze of various meshes and in different number of layers as filters for mouth spray. A suitable subject for these tests was found in an adult who was the subject of a chronic antrum and ethmoid suppuration with constant purulent discharge, in whose throat and mouth abundant *Streptococcus viridans* were constantly present. It has been noted by those who have studied the bacteriology by mouth sprays that the number of bacteria discharged is exceedingly variable when coughing efforts are made. We found that when our subject coughed with mouth wide open few bacteria were driven out, but that an explosive cough with the lips held quite close yielded quite a rich bacterial spray. A very abundant bacterial spray was obtained by first distending the cheeks with air and then, suddenly opening the lips a little, forcing the air out with a puff. The tests were made by having the subject direct such forcible expiratory efforts toward petri dishes containing blood agar at a distance of 6 inches, the face being uncovered and covered by various gauzes in different multiples.

TABLE 4
FORCIBLE EXPIRATORY EFFORTS

Number of colonies developing on blood-agar plates which were exposed at 6 inches to two very forcible expiratory efforts in which the cheeks were first distended with air and then the lips forced slightly apart with a puff.

Number of Layers of Gauze	Mesh of Gauze									
	20 × 14		24 × 20		28 × 24		32 × 28		44 × 40	
0	2,000		2,000		2,000		2,000		2,000	
1	2,000	100%	2,000	100%	1,500	75%	1,500	75%	1,500	75%
2	1,500	75%	1,500	75%	1,500	75%	800	40%	800	40%
4	800	40%	800	40%	500	50%	500	25%	80	4%
6	500	25%	200	10%	50	5%	5	0.25%	0	0.0%
8	100	5%	15	0.75%	5	0.5%	1	0.05%	0	0.0%

The results shown in Table 4 were obtained on a day when the streptococci were especially abundant. The colonies developing on the plates were practically all those of *Streptococcus viridans*.

It will be noted that the coarser gauze allowed a large proportion of the bacteria to pass through, even when 6 layers were superimposed. On the contrary, the finer gauzes removed many more of the bacteria, and when 6 and 8 layers were used almost all the bacteria failed to pass through. This test was rather severe, as the force used was

greater than that made in any spontaneous expiratory effort. Similar results were obtained when the pharynx and tongue had been smeared with a culture of *B. prodigiosus* shortly before the experiments were carried out. There appeared to be no appreciable difference between dry and moist gauze in filtering properties.

TABLE 5
STREPTOCOCCUS VIRIDANS EXPERIMENTS

Number of colonies of *Streptococcus viridans* developing on blood-agar plates when exposed to two explosive coughs with lips slightly parted

Distance from Mouth to Plate	No Gauze	Three Layers of Gauze 44 × 40		Colonies	Per Cent. Passing Through	Per Cent. Excluded
		Over Face	Over Plate			
6 inches.....	+	150		
6 inches.....	..	+	..	20	13.3	86.7
6 inches.....	+	16	10.6	89.4
1 foot.....	+	150		
1 foot.....	..	+	..	8	5.3	94.7
1 foot.....	+	12	8.0	92.0
2 feet.....	+	2		
2 feet.....	..	+	..	1	50.0	50.0
2 feet.....	+	1	50.0	50.0
3 feet.....	+	1		
3 feet.....	..	+	..	0	0.0	100.0
3 feet.....	+	0	0.0	100.0

Since 3 or 4 layers of gauze with a mesh of 44 by 40 removed most of the bacterial spray thrown with unusual force at a short distance, further tests were carried out to learn how efficient as filters of mouth spray 3 layers of this gauze would be when placed over the mouth of the person discharging the spray and over the exposed plate at varying distances, corresponding to the face of the person in the neighborhood. The plates were placed vertically as in the preceding experiment. The expiratory effort consisted of 2 strong coughs with the lips slightly parted. Tables 5 and 6 show the results of 2 such experiments, similar ones with slight variation being secured many times. The same person served in these tests as in the previous ones. When the gauze mask was over the face, very few colonies developed in the plates. When the gauze was over the plates the proportion of colonies as compared to unobstructed plates was also small, but slightly larger, because here the finer particles are dealt with. At a distance of 2 or 3 feet relatively more of the particles reaching that distance pass through, because here only very fine particles are projected. In the cases in which *B. prodigiosus* was smeared over the pharynx and tongue fewer colonies developed in plates placed behind gauze obstruction. This is probably because the bacteria were less thoroughly distributed in the

Our experience with masks has been principally confined to their use to protect attendants on the sick from infection. They have been used not only by nurses, but by physicians in their work while taking cultures from throats, doing intubations and examining chests. The mask on the face interferes with putting the hands to the mouth and nose and so indirectly becomes a source of safety to the individual whose hands are apt to be contaminated in her work and who thoughtlessly may put them to the face. We have also used masks over the faces of mothers while nursing their babies when either one has been infected by diphtheria or has been a diphtheria carrier.

The employment of gauze masks over the face to prevent the transfer of infections to others was thoroughly worked out and practically applied by Capps⁹ in Camp Grant. He used masks to prevent cross infections in ambulances and in the admission rooms and wards of the hospital. Similar use of masks has since been generally adopted in army and navy camps and in many civil hospitals. The intelligent use of gauze masks and other measures may be instituted equally well in private families. Many family epidemics might be limited by such means. In all instances in which infections locate in the respiratory tract and in which the infectious agent is discharged in mouth spray it is reasonable to protect those about the patient by masks of gauze. With efficient and conscientious masking, carriers of diphtheria bacilli and other pathogenic bacteria might safely be allowed a large degree of freedom.

We have noticed a considerable reduction in cases of rhinitis, tonsillitis, and pharyngitis among our nurses since masks have been worn. Endeavors to limit droplet infections should not prevent equally energetic efforts to close other channels of spread of infectious materials. The use of face masks should not give an unwarranted feeling of security to those employing them and lead to neglect of the measures which prevent carriage of infectious materials through other agents. Emphasis must still be laid on proper sterilization of eating utensils, destruction of all infectious discharges, avoiding all contamination of foods and special care regarding the washing of the hands every time the sick are handled.

CONCLUSIONS

Droplet infection comes into play whenever an individual with pathogenic organisms in the mouth gets into close contact with another

⁹ Jour. Am. Med. Assn., 1918, 71, p. 448.

individual. Sneezing and suppressed coughing are most apt to produce abundant droplet spray.

Gauze will filter bacterial spray from air. Its efficiency is in direct proportion to the fineness of mesh and number of layers employed. Three layers of gauze with a mesh of 40 threads or more will remove almost all bacteria-carrying droplets. Occasional fine droplets pass through.

Gauze masks appear from clinical data to prevent infection through mouth droplets. They are useful when worn for protection by attendants on the sick, and also when worn by the infected individual to prevent contamination of his surroundings.

The use of masks should not lead to neglect of measures calculated to prevent transfer of infectious materials by other means than by droplet spray.

THE CONNECTION OF MILKSICKNESS WITH THE POISONOUS QUALITIES OF WHITE SNAKE- ROOT (*EUPATORIUM URTICAEOFOLIUM*)

WALTER G. SACKETT

From the Department of Hygiene and Bacteriology, University of Chicago

INTRODUCTION

Since the pioneer days of 1840, when hundreds of acres of our most fertile river-bottom lands and wooded slopes were still uncleared, some animal husbandmen have associated the common white snake-root, *Eupatorium urticaefolium*, with a disease of cattle, sheep and horses, variously designated as "milsickness," "milk sick," "staggers," "trembles," "alkali" and "slows." Later opinion has tended to confirm this view, but much of it has been little more than the expression of the personal impression of untrained observers.

As early as 1839, we have the record of Rowe¹ who fed the plant to cattle with fatal results.

Barbee,² in 1840, after searching for possible causes of the disease, concluded: "Nor have any circumstances in the history of 'Milk-Sick' in these districts (Ohio) enabled me to approach nearer to a discovery of its cause than that it probably has a vegetable origin. By some it would be deemed altogether unnecessary to offer anything in the support of the supposition of the vegetable origin of the disorder. This is the most popular theory and has more facts to sustain it than that which contends for a mineral poison." He had noticed that cows which were enclosed in woodland pastures developed trembles and died. His observations led him to believe that the two plants which were probably responsible were white snake-root and poison oak (*Rhus toxicodendron*). He fed a decoction of the first to a dog which showed characteristic symptoms and died in 3 hours; to another he gave a decoction of the latter plant with results similar to the first, except that the animal died in 2 hours. Barbee states that an acquaintance of his, Dr. Owen, had fed a decoction of *E. ageratoides* (*urticaefolium*) to a calf which developed trembles and died in a few hours. A fact of additional interest is that Dr. Owen had received specimens of plants identified as *E. ageratoides*, from farmers in Indiana who claimed that this species was responsible for trembles. Some argued that it could not be the cause of the trouble for, were that the case, all cattle which ate it should die, and this was not true. In defense of his position, Barbee advanced the idea of individual resistance among the animals, asserting that "the vital powers may resist the poisonous agency in any quantity."

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¹ Ohio State Jour., Sept. 6, 1839.

² Western Jour. Med. and Surg., 1840, I. p. 178.

In June, 1855, Vermilya³ of Ashland Co., Ohio, confined 200 sheep in a pasture where white snake-root was growing along with other weeds and grass. He noticed that the animals did not eat it as long as there was any grass available. In the course of time, several sheep developed trembles, and after 6 or 8 had died and 5 others were affected, he took them away and all recovered. An old sheep was given the run of this same pasture, but refused to eat the snake-root even when tempted to do so by salt sprinkled over the leaves; it licked off the salt, but would not taste the plant. In October of the same year, an old mare was turned into this enclosure but ate the weed only when it was mixed with oats; on the 3rd day she ate some clear and died on the 4th day. One year later, Vermilya fed small quantities of it to a 7-year old mare daily until she refused it; trembles appeared and the horse died 11 days after the feeding was begun. Regarding the attitude of livestock toward the plant, this observer says, "I think that nothing will eat it when they can get plenty of other food."

It is worthy of note, in passing, that in these experiments, *E. ageratoides* was poisonous both in June and October, a point of interest, since some plants are said to be much more dangerous at one period of their growth than at another.

In summing up his views on milksickness, Vermilya in 1858 says: "The malady prevails to a considerable extent in many parts of our country, more particularly in new and uncultivated lands. Horses, sheep and cattle are affected by it, as well as people; indeed, people are not afflicted by it except by the use of milk taken from a cow which is diseased. It sometimes appears in old settlements, but if so, it is owing to a season not furnishing sufficient food for cattle, by which they are driven to eat articles that, in other seasons, when food was plenty, they would reject. Thus, the past season, when food was scarce, this disease prevailed considerably in various parts of the state. We have a cause that will produce the effects indicated by this disease. A species of *Eupatorium*, commonly called white snake-root, will produce all these effects. In materia medica, it is used for that purpose. It grows in shady, moist land, and if food is plenty, cattle will not eat it. When pinched by hunger, they will."

One of the most interesting cases on record is that reported by Jerry⁴ of Madison Co., Ill. In June, 1860, he collected what he supposed to be common nettle for greens. His wife cooked them and he was the first one to taste them. He noticed at once a peculiar flavor and odor, quite different from that of nettle greens and immediately suspected that he had gathered the wrong plant. None of the other members of his family ate any, and they all remained well. The next day, however, Jerry was taken violently ill with symptoms which were identical with those of milksickness. Recurring attacks were experienced, and he did not recover completely for five years.

In this respect, the history of the case corresponds very closely with those said to be acquired through the use of milk.

Later, Jerry fed a decoction of the unknown plant, which had sickened him, to his dog, and the latter became violently ill as a result. Some seven years later the "greens" were identified by Enon Sanders of Edwardsville, Ill., as *E. ageratoides*.

³ Ohio State Board of Agr., 13th Ann. Rept., 1858, p. 670.

⁴ Med. and Surg. Report, 1867, p. 270.

Observations of this sort by the early settlers could be duplicated almost ad infinitum, but the few examples cited serve to give an idea of the importance which was attached to poisonous plants, and more especially to white snake-root, as the cause of trembles.

The history and progress of the disease seem to have followed much the same course in both man and the lower animals, a fact which might point to a common origin or to an inter-relation of causal agents.

A most excellent historical sketch and bibliography of milksickness have been given by Jordan and Harris,⁵ and there is no need for unnecessary repetition at this time, except for a few of the more salient points which are of more or less general interest.

HISTORICAL

The fact appears to be well established that the disease existed in North Carolina as early as 1778, and that by 1825, as civilization moved westward, it was well known to the settlers of Tennessee, Kentucky, Ohio, Indiana, Illinois and Michigan.

Since 1840, there has been a gradual but marked falling off in the number of cases until at the present day, it is a relatively rare occasion for a physician to be called to wait on a patient with milksickness. Whereas, in the early days there was hardly a medical man who had not had first-hand experience; today, it is the exceptional practitioner who has ever seen a case. Occasionally, however, even nowadays, outbreaks occur such as those reported by Jordan and Harris^{6, 5, 7} in New Mexico, in 1907, in Altamont, Illinois, in 1908; by Walsh⁸ at Morris, Ill., in 1908 (7 cases); by Clay⁹ in Vermilion, Clay and Wayne Counties, Ill., in 1913 (17 cases), and by Schwarze¹⁰ near Atwood, Ill., in 1917.

As one begins to analyze the data at hand in an attempt to account for the almost complete disappearance of trembles and milksickness, two facts stand out very prominently:

In the first place, in the pioneer days when the disease was most prevalent this entire region was heavy timberland, and in all probability cattle roamed unrestrained. The native vegetation, including poisonous species, presumably

⁵ Jour. Infect. Dis., 1909, 6, p. 401.

⁶ Jour. Am. Med. Assn., 1908, 50, p. 1665.

⁷ Science, 1909, N. S. 29, p. 1010.

⁸ Ill. Med. Jour., 1909, 15, p. 422.

⁹ Ill. Med. Jour., 1914, 26, p. 103.

¹⁰ Jour. Am. Vet. Med. Assn., 1918, 53, p. 236.

was very dense and every possible opportunity was offered to the stock to feed on these plants. Under such circumstances, trembles developed at an unprecedented rate.

In the second place, as the land has been cleared, drained and brought under cultivation, which operation is still going on, the natural range for cattle has become less and less each year, and will continue to do so each succeeding year. With the gradual elimination of the native pasture, the opportunity for ingesting poisonous plants has been reduced, with the result that trembles and milksickness have disappeared at a rate commensurate with the amount of clearing.

Another fact which probably plays a part in the decrease in the number of cases of trembles and milksickness is better diagnosis; we have every reason to believe that formerly many ailments were pronounced milksickness which today would be given an entirely different classification.

It will probably be found that more than one species of plant is capable of exciting the symptom complex termed "trembles." Further study may show different group complexes, each separate and distinct from the others and characteristic of a particular disease specific for a single species of poisonous plant.

ETIOLOGIC FACTORS

The number of poisonous plants thought to affect milk in one way or another, either by increasing or decreasing the flow, or by imparting some disagreeable flavor, objectionable color or deleterious quality is relatively large. According to Long¹¹ the list for the United Kingdom includes 31 different genera. Of these, *Colchicum autumnale*, when fed to goats, has made the milk poisonous for infants; *Narthecium ossifragum* is alleged to have poisoned a cow so that its milk killed a cat; *Euphorbia lathyris* when eaten by goats is said to impart its poisonous qualities to the milk.

In addition to *Eupatorium urticaefolium* and *Rhus toxicodendron*, already mentioned in connection with trembles, several others have been named as standing in a possible causal relation:⁵ "*Lobelia inflata* (Indian tobacco), *Bignonia capriolata* (cross vine), *Apocynum cannabinum* (Indian hemp or Indian barley), *Caltha palustris* (marsh marigold), *Euphorbia esula* (spurge), *Aethusa cynapium* (fool's parsley), *Psedera quinquefolia* (Virginia creeper), *Symphoricarpos orbiculatus* (Indian current), *Cicuta maculata* (cowbane), and *Bigelovia rusbyi*" (now probably *Chrysothamnus bigelovii*), rayless goldenrod or rabbit brush of Colorado and New Mexico.

Poisonous mushrooms were suggested by Winans,¹² in 1840, as a possible cause of the disease. Johnson¹³ in 1866 held the same view, although admitting that he had little experimental proof to fortify his position. Twenty years previously, he had mixed the expressed juice of a mushroom with milk and sugar, and when this was fed to flies, chickens and a cat, it resulted fatally. In his own words he says: "I am of the opinion that this poisonous agent is the mushroom, and whilst I am not able to demonstrate the fact from an actual test, still it is the only conclusion justified by the fact at which I have been able to arrive."

¹¹ *Plants Poisonous to Livestock*, 1917, p. 99.

¹² *Western Jour. Med. and Surg.*, 1840, p. 191.

¹³ *Atlanta Med. and Surg. Jour.*, 1866-67, 7, p. 289.

Slack,¹⁴ in 1854, maintained that trembles was due to a parasitic fungus like ergot which infected the vigorous swamp grasses of the lowlands, and that cattle which were pastured in such localities ate the poisonous principle along with the grass seed. To the active constituent he gave the name, "Ergdeleteria." In defense of his position he says, "the cause has been removed by clearing and cultivating the land, and draining the swamps. Wherever drainage and cultivation are vigorously pushed, the disease is no longer found; because the strong pasture grass having air and sunlight, the nidus of the fungus seeds is not furnished and the fungus, which is a poison parasite, will not grow and ergotise the seed of the grass."

It is a matter of experience that just such treatment of the land as is described above has also reduced the amount of white snake-root very materially.

Nagle¹⁵ was also an exponent of the ergot theory (1859). His conclusions were based, in part, on limited feeding experiments in which grass from swamp was cut and fed to a cow. In the course of time trembles was produced and death followed. The meat from these animals, when fed to dogs, killed them. Nagle's description of the grass, leaves little room to doubt that there was a parasitic fungus present; in fact, it was confirmed by microscopic examination. The question hinges on whether his cows developed typical symptoms of trembles, or of ergot poisoning.

While the poisonous plant theory appears to have been the one most generally accepted as the cause of the disease, numerous other views have been advanced to explain its origin.

The mystic miasma emanating from swamps, which for years veiled the secret of malaria; the web of an innocent insect; the dew on the grass; a yeast; impure air; diseased soil; and even "remaining in the timber over night," all have received their share of consideration. A mineral, "rising out of the ground and collecting on the leaves of plants in yellow droplets" was found by the writer regarded as the cause in Tennessee as late as July, 1918. This deposit was claimed to be especially noticeable "of a morning" when the dew was heavy and if the plants were growing in the proximity of a spring. The possible relation of impure water has not been lost sight of; mineral salts which had accumulated in excess in stagnant pools from which the cattle drank have been suggested; animals are said to have licked the soil and obtained poisonous minerals in that way; minerals of a metallic kind such as those of arsenic, copper, mercury, cobalt, barium and aluminum believed to exist in the native vegetation in toxic amounts, have been exploited by their respective exponents.

During the past year, Curtis and Wolf,¹⁶ Marsh and Clawson,¹⁷ and Moseley¹⁸ have published the results of experiments which point

¹⁴ Western Lancet, Cinn., 1854, 15, p. 140.

¹⁵ Nashville Jour. Med. and Surg., 1859, 17, p. 289.

¹⁶ Jour. Agr. Research, 1917, 9, p. 397.

¹⁷ Jour. Agr. Research, 1917, 11, p. 699.

¹⁸ Med. Rec., 1917, 92, p. 428.

quite conclusively to *Eupatorium urticaefolium* as the true cause of trembles.

The more recent conceptions are of sufficient importance to warrant our giving them greater consideration than some of the earlier experiments, and therefore they will be discussed more in detail in the following pages.

RECENT EXPERIMENTAL WORK

Moseley,¹⁹ in 1905, was the first to undertake anything in the way of a systematically organized study of trembles, according to our modern idea of what such experiments should be. He prepared both a milk infusion and a water decoction from fresh snake-root leaves and stems, and fed this to cats and rabbits. Both animals showed symptoms of the disease, but only the cats died as a result. Rabbits fed with the green plant developed trembles and died after 3 days. The viscera and meat from these rabbits were given to cats, trembles appearing in all, but only 1 died. A dog was given the aqueous extract and the chopped leaves and stems of plants along with milk and other food; in less than 24 hours, the dog was seized with violent trembling but recovered. A sheep was feed the green plant, of which it consumed approximately 29 ounces; it remained normal until the 3rd day when it manifested characteristic trembles and prostration; it died the next day; the necropsy showed both kidneys to be greatly enlarged. An aqueous decoction was injected subcutaneously into a rabbit, as a result of which trembles followed, but not death; subsequently, the animal was fed fresh snake-root which killed it in 5 days. In commenting on the behavior of the poison, Moseley states that his experiments with both rabbits and cats indicate that these animals may acquire some degree of tolerance for the poison.

Crawford,²⁰ in 1906, prepared an aqueous extract from both dried plants and those preserved in water to which sufficient chloroform was added to prevent fermentation. The extract was either fed or injected subcutaneously into rabbits, cats and dogs; 4 rabbits died as a result. He also injected the water extract of the ash from a dried plant into a rabbit with doubtful results. A lamb weighing 25 kg. was fed 58 gm. of fresh material with no other effect than a slight diarrhea. Crawford, himself, took the extract from approximately 300 gm. of the fresh plant without experiencing any of the symptoms of milksickness.

As a result of his experiments, he concludes that "it certainly cannot be said that it has been proved that milksickness is due to any constituent of *Eupatorium ageratoides*." Notwithstanding the fact that 4 of his 13 experiments gave positive results, he is disposed to dismiss Moseley's work with the challenge, "that it cannot be said that Moseley has even proven *Eupatorium ageratoides* to be a poisonous plant, much less the cause of trembles."

Moseley,^{19a} in 1909, claims to have produced the same results in rabbits by feeding them aluminium phosphate as by giving them white snake-root. He has found considerable quantities of this salt both in the leaves of *Eupatorium urticaefolium* and in the stems of *Isocoma heterophylla* (rayless goldenrod). Aluminium was found in the milk of a cow fed with white

¹⁹ Ohio Naturalist, 1906, 6, p. 463.

^{19a} Moseley, E. L.: Med. Rec., 1910, 77, p. 620.

²⁰ Bulletin No. 121, Part I, Bu. Plant Industry, U. S. Dept. Agr., 1908.

snake-root and this milk produced trembles in cats and rabbits. Aluminium was found in the urine, liver, kidneys and muscle of rabbits fed snake-root and the meat, whether raw or cooked, produced trembles in cats. Therefore, Moseley concludes that trembles and milksickness are due to aluminium phosphate and that animals get this substance from white snake-root in the Central States and from the rayless goldenrod in New Mexico.

Later²⁰ he examined the butter from a farm where milksickness had occurred in the family and found it to contain 0.02% of aluminium phosphate. When this butter was fed to cats, it produced trembles in a few days. Further feeding experiments were carried out on cats with aluminium phosphate, and it was found to be most effective when given with fat meat, and less so if mixed with lean meat; administered in milk it was less active than with fat meat, while with vegetables it had no perceptible action. The use of sodium bicarbonate as a remedial measure as recommended by Walsh⁸ was attended with beneficial results, and hence Moseley believed that "a part of the injury to the system produced by the aluminum was due to its producing a tendency to acidity."

Jordan and Harris^{5, 6, 7} during an outbreak of so-called milksickness in New Mexico, isolated an aerobic, spore-forming bacillus in pure culture from cases occurring in both man and the lower animals. At the time, they thought that this micro-organism might be the responsible agent, and accordingly named it "*Bacillus lactimorbi*." Subsequent experiments with laboratory animals, in which pure cultures of the organism were both fed and injected, have produced little or no effect, and they concluded finally: "Taken as a whole the facts do not surely indicate that a special micro-organism is the cause of milksickness or trembles."

Clay⁹ of Hoopeston, Ill., after losing a large number of cows and horses from trembles prior to 1914, procured two young cattle and one sheep that were healthy and that had not been in wild pasture. They were confined in a barn-lot and required to eat *Eupatorium urticaefolium* cut fresh at each feeding. All were dead within 3 days, the symptoms being identical with those of animals which had died previously on the natural range. He believes that the disease is due to an intoxication rather than to an infection, since in the 17 cases in man which he attended in 1913, he was unable to find *Bacillus lactimorbi* of Jordan and Harris either by cultural methods or by direct microscopic examination of the blood or urine.

Marsh and Clawson¹⁷ fed sheep and cattle with both dried and fresh *Eupatorium urticaefolium* at Washington, D. C., and Beecher, Ill., in 1914. Their results show conclusively that the plant is toxic, and that its ingestion is accompanied by a definite symptom complex resembling that described for trembles. They believe that the poison has a cumulative effect, and that the plant loses a large part of its toxicity in drying.

Curtis and Wolf,¹⁶ in 1916, fed fresh *Eupatorium urticaefolium* to 15 head of sheep; all of these developed trembles; 14 died and 1 recovered. Healthy sheep confined with the affected ones failed to show the disease; this fact is very properly considered as indirect evidence against the infectious nature of the disease. No harmful effects resulted from feeding aluminum phosphate with grain and hay; salt and soda administered with the food were without remedial value.

Within the past year (1917) Moseley¹⁸ has prepared an ether extract from the leaves of white snake-root. The plants were gathered in a woods which had served as pasture for a cow whose milk and butter are said to have

caused milksickness in 3 members of a family, 2 of whom had died. He states that nearly all of this extract is a resin, and that when taken with food by rabbits, it affects them similarly to snake-root. The same symptoms were produced in a guinea-pig. Cats show some of the symptoms of trembles, but as a rule they will not eat enough to kill them.

The inconclusive character of some of the experiments seemed to warrant a further examination of the subject.

THE GENUS EUPATORIUM

Name of Genus.—This genus is said to be christened²¹ Eupatorium after the ancient king of Pontus, Eupator Mithradates VI.

BOTANICAL CONSIDERATION

Species Description.—Eupatorium urticaefolium, Reichard (Eupatorium ageratoides, L. f.—white snake-root²²).

Heads 8-30 flowered; involucre bracts nearly equal, in one row or but a very few of the outermost shorter. Plants smooth, branching, 0.5-1 m. high; leaves broadly ovate, opposite, long petioled, triple nerved, not resinous, dotted pointed, coarsely and sharply toothed, thin, 7-12 cm. long; corymbs compound, flowers pure white. Rich woods, not rare.

This species thrives best in a rich, moist soil, rather light in texture, such as is found in our shaded coves or ravines and moist woodlands. Protected by the trees, the plant seems to find optimum conditions for its growth in the heavily shaded, moist areas in the vicinity of a stream. We rarely find it growing in the open, unless it is where the land has been recently cleared of timber and where an abundance of moisture is still present. Bright sunshine causes rapid wilting, and accordingly, it disappears early from unprotected areas.

Eupatorium urticaefolium is reported as occurring in the states of the United States east of the Rocky Mountains, except in Kansas, Oklahoma and Texas. West of the Rockies, its presence has not been observed up to the present time.

Number of Species.—The number of species of Eupatorium which have been observed and described in the different parts of the world, is rather astonishing, considering the fact that most of them are only common weeds, and very unpretentious at that. In the neighborhood of 400 different kinds have been noted, all but about a dozen of which are natives of the Western Hemisphere, and most of these are to be found in the warm temperate or tropical regions. Some 50 have been reported as used in one way or another in the arts and industries.

DISCUSSION OF THE ACTIVE CONSTITUENTS OF EUPATORIUM SPECIES OTHER THAN E. URTICAEFOLIUM

According to Hager,²³ "E. perfoliatum L. is native to American Southern states. The plant is used as a source of bitters. It is said to contain a glucosid, which is also called Eupatorine, a nonglucosidal principle, 0.1% of

²¹ Sprengel, Kurt: Versuch einer pragmatischen Geschichte der Arzneikunde, 1800, Pt. 1, p. 634.

²² Gray's New Manual of Botany, 7th Edit., Rev. 1908.

²³ Handbuch der Pharmaceutischen Praxis, 1900, 1, p. 1069.

volatile oil, gallic acid, tannic acid, etc., and the flowering plants an alkaloid. The leaves are used, as well as the extract.

"Also *E. purpureum* L., trumpet weed, purple boneset or gravel root contains a little volatile oil and the glucosid Euparine, $C_{12}H_{11}O_4$."

Several members of this genus have been used for the volatile oil which they contain; two have been employed as a source of indigo; two as substitutes for hops and one in place of tobacco. The leaves and blossoms of *Eupatorium perfoliatum* and *E. purpureum* contain bitter principles to which their reputed medicinal value has been ascribed.

Eupatorium perfoliatum, L.—The American Indians appear to have been the first to utilize *Eupatorium perfoliatum* or common boneset as a medicinal plant.

Anderson,²⁴ in 1813, reported its use among the native tribes as a successful remedy in the treatment of "intermitting and remitting" fever.

Stevens²⁵ and Hosack²⁶ reported that this species was known to be popularly used for the cure of malaria in 1803 and it was advocated also in the treatment of yellow fever.

Wilkins²⁷ reports successful use of the leaves in the treatment of tapeworm.

Peterson²⁸ believed that this plant contained some principle peculiar to itself and different from quinin, as had been previously suggested, to which its tonic and emetic properties were due. Although Peterson was unsuccessful in isolating the active constituent in a pure state by treating the leaves with water and subsequently extracting the aqueous residue with alcohol, he proposed the same "Eupatorin" for the bitter residue.

In 1854, Bickley²⁹ obtained a yellow, slightly crystalline substance, extremely bitter and nauseating from a cold aqueous infusion of the leaves which was subsequently concentrated, dried and treated with boiling 95% alcohol. He did not proceed further to determine the nature of the yellow substance.

Collier and Parsons³⁰ made an analysis of *E. perfoliatum* in 1879 and found a bitter principle which is described as a brown uncrystallizable substance soluble in water and alcohol, insoluble in ether.

Latin,³¹ in 1880, obtained a bitter principle which yielded reducing sugars on hydrolysis. He stated that this was probably of glucosidal nature and that it was soluble in alcohol, ether, chloroform, boiling water and concentrated acids.

Franz³² found a bitter principle in the leaves which was nauseous. Hydrolysis experiments led him to believe that what he had obtained was a glucoside.

In 1892, Shamel³³ separated what he believed to be the active ingredient in pure form. The bitter principle occurred either as a yellow resinous mass or as a yellow powder, which, under the microscope, showed globular masses of needle-shaped crystals. These were soluble in dilute nitric acid, and when the nitrate was allowed to crystallize "beautiful prisms and six-sided plates" were obtained. "An aqueous solution of these crystals injected into mice killed them in a few hours."

²⁴ A Dissertation on the *Eupatorium perfoliatum*. May 4, 1813.

²⁵ Med. Repository, VII, 12.

²⁶ Med. Essays, III, p. 434.

²⁷ Am. Jour. Phar., 1874, 46, p. 295.

²⁸ Am. Jour. Phar., 1851, 23, p. 206.

²⁹ Am. Jour. Phar., 1854, 26, p. 495.

³⁰ Am. Jour. Phar., 1879, 51, p. 342.

³¹ Am. Jour. Phar., 1880, 52, p. 392.

³² Am. Jour. Phar., 1891, 60, p. 77.

³³ Am. Chem. Jour., 1892, 14, p. 224.

The roots of this plant were examined the same year by Kaercher³⁴ who secured an amorphous bitter substance soluble in chloroform but which gave no reaction with either alkaloidal or glucosidal reagents.

In attempting to repeat the work of Shamel, Walter,³⁵ in 1900, discovered that the crystals, which the former had obtained by treating his yellow residue with nitric acid, and which he believed was the nitrate form of the active ingredient, were nothing more than crystals of pure oxalic acid such as might be expected to result from treating tannic acid with nitric acid, and which in Shamel's preparation had probably originated from the tannin that was present as an impurity. In the light of this disclosure, it would appear that the mice which Shamel injected succumbed to oxalic acid, rather than to some other form of poison present in the active principle.

The active ingredient as obtained in a purified form by Walter was a light reddish-brown residue, extremely bitter, having the formula $C_{35}H_{58}NO_{10}$. This was neither fed nor injected into animals, so we have no data on its poisonous properties.

Reasoning by analogy from other bitter principles which are known to be active ingredients of certain drugs, we are naturally led to the conclusion that, here too, the active principle resides in the bitter substance, but the fallacy of such reasoning without animal experimentation is too obvious to need further comment.

Eupatorium purpureum.—Siggins,³⁶ in 1888, obtained an ether soluble substance from the leaves of this species which he believed to be a glucosid with a bitter taste.

In the same year Trimble, Ray and Eberhardt³⁷ extracted a yellow, resin-like uncrystalline substance from the roots of this plant by means of ether, but it gave none of the tests for either alkaloids or glucosides. It was named Euparin, as suggested by Lloyd³⁸ and given the formula $C_{12}H_{11}O_3$.

Manger,³⁹ in 1894, confirmed the work of Trimble.

From the analyses made on *E. purpureum* it appears that the leaves contain a bitter principle, glucosidal in character, and that the roots yield a crystalline nonglucosidal substance, Euparin.

THE PRESENT WORK

Frequent reference is made to the fact that trembles is most prevalent among cattle which are pastured on moist, shaded, rich land. This is where white snake-root finds conditions adapted to its growth.

We read that the disease is always worse in a dry season or late in the fall when feed is short and poor. This can be easily explained, for during such times the native grasses would be insufficient for the animals' needs, and they would eat anything green, regardless of whether it was snake-root or red clover.

³⁴ Am. Jour. Phar., 1892, 64, p. 510.

³⁵ Proc. Am. Phar. Assn., 1900, 48, p. 216.

³⁶ Am. Jour. Phar., 1888, 60, p. 225.

³⁷ Am. Jour. Phar., 1890, 62, p. 73.

³⁸ Am. Jour. Phar., 1876, 48, p. 331.

³⁹ Am. Jour. Phar., 1894, 66, p. 120.

On the other hand, very little of the disease is experienced if the season is wet. Under such conditions, the natural range would be good and sufficient for the animals' demands, and there would be no occasion for them to eat poisonous weeds.

It is asserted that stock in one pasture or locality are affected, while the animals in an adjoining or neighboring tract are not, yet to the casual observer conditions are identical. The observations of the writer lead him to believe that a more careful study of the forage possibilities in the two localities would explain this point, for just such a circumstance was called to his attention last fall. An examination of one pasture (Morris milksick) showed practically nothing but weeds, while just across the road (disease free) there were plenty of the same kinds of weeds, but, in addition, abundant blue grass. Manifestly the cattle feeding on the one side of the road would be compelled to eat snake-root, while on the other they would not. Extracts of plants from both areas have been shown to be equally poisonous, so that the failure of the animals to develop trembles on the one tract cannot be charged to a lack of poisonous properties in a part of the plants, but rather to the failure of the cattle to eat the snake-root where grass was plentiful.

Out of such assertions as the foregoing, the belief has grown up that snake-root from one locality is poisonous while that from another is not. Neither the truth nor the falsity of this claim can be established at present.

· COLLECTION OF MATERIAL

In order to determine whether such expressions as these were merely opinions, or whether experimental evidence could be produced that would justify these utterances, we endeavored, with the cooperation of the state experiment stations, to obtain green plants from widely separated regions. Unfortunately, requests for this material were not sent out until the middle of October, 1917, and by that time, many localities had been visited with heavy, killing frosts, so that the range of territory represented was not as large as we had hoped for. However, specimens were received from Alabama, Iowa, Massachusetts, Minnesota, Missouri, Ohio, Pennsylvania, Tennessee and Wisconsin.

Through the courtesy of Dr. Walsh of Morris, Ill., we learned of a most interesting "milksick" area, a moist woodland pasture, bordering a stream, where *Eupatorium* was growing abundantly and which has a definite history. The land in question lies on either side of the main traveled road, and it was alleged that cattle which were allowed to browse on the side of the highway invariably contracted milksickness, and some animals had died, whereas if they were permitted the range of the other side, no evil consequences ensued. The owner was so confident that his observations were borne out by facts that he had caused a limited area on the bad side to be fenced with

barbed wire so that his cows could not have access to it. The vegetation on both sides was much the same, except that the tree growth on the milksick portion was perhaps somewhat denser, and the ground here being naturally more shaded, there was comparatively little grass in proportion to the luxuriant crop of weeds. On the disease-free side, the pasture was more open, possibly the trees stood farther apart, and the grass was much more abundant, that is to say, it was a very much better pasture. The *Eupatorium*, if anything, was more plentiful here than across the road. Having seen the two pastures, with their respective flora, it seemed almost self-evident why the one was dangerous for stock, while the other was relatively safe, for in the first case, the animals had been forced to eat weeds, snake-root being among those present, whereas, in the second, there was plenty of good wholesome grass which they quite naturally preferred, and so did not molest the *Eupatorium*.

The first collections were made Oct. 20, 1917, from the milksick area only, and at that time, the plants were just coming into flower. Two weeks later, when blossoming was practically over, a second lot of material was secured from both areas. Entire plants, including roots, stems, leaves and blossoms, were gathered; they were expressed to Chicago, and immediately on arrival, the roots were removed, washed free from soil and dried; the leaves and flowers were stripped from the stems and each air-dried separately, in order to prevent any possible injury from molding. While the plants were being collected, we were constantly on the lookout for anything that might be present on the foliage in the way of a parasitic fungus, since there was the possibility of some lower form of this sort having a causal relation to milksickness, but nothing of the kind was ever observed either on Illinois material or that from elsewhere.

GREENHOUSE MATERIAL

Some fifty roots, from Illinois stock and elsewhere, were set in a greenhouse in the late fall of 1917. This supplied green material for feeding during the winter.

PREPARATION OF THE CRUDE DRUG

Whenever the condition of the plants made it possible and the size of the sample justified it, the roots, stems and leaves were dried separately, and as soon as they were thoroughly air-dry, were prepared for extraction by reducing them to a No. 30 U. S. P. powder by grinding in a suitable mill and finally by passing them through a 30 mesh sieve. Such powders were stored in glass stoppered bottles in subdued light.

PREPARATION OF EXTRACTS

During the course of this study we have employed several different methods of extraction, all having for their ultimate object, the separation of an active, poisonous principle. As solvents we have used 95% alcohol, 70% alcohol, physiologic salt solution (0.85% NaCl in distilled water), and a mixture of ether, chloroform and ammonia.

ALCOHOLIC EXTRACTS

Fifteen gm. of the No. 30 powder, an amount slightly in excess of the quantity of the leaf powder required to kill a rabbit as shown by feeding experiments, were moistened with 95% alcohol and packed firmly into a glass percolator of approximately 350 cc capacity. Enough 95% alcohol was then

added to saturate the mass and leave a thin layer on top. It was allowed to macerate in this condition for 48 hours. At the end of this time, 150 cc of alcohol were added and the percolation allowed to proceed at the rate of about 60 drops per minute. When this alcohol had all run through, more was added and the percolation continued until the drug was exhausted or until the percolate had only a very pale green color; usually 200 cc of alcohol were sufficient to accomplish this.

From this point, two different procedures have been followed to reduce the tincture, obtained above, to the consistence of a heavy paste: Either the alcohol was allowed to evaporate in the breeze from an electric fan, or the alcohol was distilled off under reduced pressure at a temperature under 35° C., the thick liquid transferred to an evaporating dish by means of a small quantity of 95% alcohol and the whole finally concentrated by the electric fan.

The solid extracts prepared by either of these methods are black in color, waxy or gummy in character, of a bitter taste which is very persistent, slightly pungent and possessing an aromatic or resinous flavor.

SALT SOLUTION EXTRACT

The salt solution extract was prepared in much the same way as the alcoholic, except that the period of maceration was reduced to 6 hours and the percolation carried out with physiologic salt solution until the percolate was practically colorless. This yielded a dark brown liquid of approximately 200 cc in volume, which was reduced to a thick syrup by boiling under reduced pressure at a temperature below 40° C. The heavy, dark brown liquid was transferred to an evaporating dish from the distilling flask with a minimum quantity of distilled water, and evaporated by means of an electric fan to a resinous, doughlike mass, shiny and almost black in color. The quantity of solid material which was yielded to salt solution was considerably greater than that obtained with 95% alcohol as the menstruum. The residue was rather pungent, with a tendency to pucker the tongue, rather than possessed of a bitter taste, and with a slight aromatic flavor.

ETHER-CHLOROFORM EXTRACT

For the extraction with ether, chloroform and ammonia, an infusion method was employed. This consisted in treating 15 gm. of the No. 30 powder in a 500 cc Erlenmeyer flask with a mixture, composed of 115 cc of ether and 35 cc of chloroform for 10 minutes in a shaking machine. Five cc of a 10% ammonia solution were then added to the mixture and the shaking continued for 2 hours. At the end of this time, the infusion was carefully poured off through absorbent cotton and then filtered through paper (S and S No. 597). This gave a beautiful, clear, dark green liquid which was allowed to evaporate spontaneously, yielding a very dark green residue, waxy in character, slightly bitter, somewhat aromatic, but with no decided flavor. The bulk of the residue was very much less by this method of extraction than with either of the two preceding, amounting in many cases, where leaf powder was used, to less than one-half gram. The bulk of a residue, where this is to be fed in its entirety to laboratory animals at a specified rate and in a given length of time, is an item which must be taken into consideration in preparing these crude extracts, since more or less difficulty is apt to be experienced in administering these even if given in pills or capsules.

FEEDING EXPERIMENTS

The work of Marsh and Clawson,¹⁷ and more recently that of Curtis and Wolf¹⁶ indicate that sheep and cattle fed on *Eupatorium urticaefolium*, experimentally at least, develop trembles and eventually may die. That there is some rather intimate causal relation between this weed and the disease, there can be no longer any reasonable doubt, and on this ground, there is ample reason for including this malady among those which are considered as being due to food poisoning. Under this classification, however, there are several possibilities.

In the first place, specific pathogenic micro-organisms may be present on the plants which for some unknown reason have selected this particular species as a host, and when ingested by the lower animals, they develop a toxin which produces the symptoms observed. It will be recalled that *B. lacti-morbi* was mentioned at one time in this connection by Jordan and Harris;⁵ however, at present, they are disposed to attach less weight to their findings than formerly.

In the second place, there is the question of micro-organisms growing in and on the plants and there producing poisonous substances in the tissues either thru their own metabolism or as a result of cell destruction and disintegration in the plant. If this condition existed, we should almost certainly expect to find some external evidence manifesting itself as a plant disease. While no less than nine different parasitic fungi have been reported by Farlow and Seymour on this species of *Eupatorium*, the writer has taken careful cognizance of this point when collecting material, and nothing has ever been observed.

The third possibility which may be offered in explanation of this relation, and the one with which the present work is concerned, maintains that there is present in the plant itself some active poisonous principle, perhaps in the nature of an alkaloid or glucosid, which may be responsible for the trouble. That this contention is well founded, at least in so far as it pertains to rabbits, is borne out by feeding experiments conducted during the past 6 months.

Rabbits, guinea-pigs and cats have been used as the experimental animals. The first have been fed the green plants, the dried powders and the different extracts; the guinea-pigs received the last two only, and the cats were given the viscera and meat from the rabbits that died.

The fresh Eupatorium was fed by itself as long as the animals would eat it, and when they refused, it was chopped very fine and mixed with either ground carrots or celery or cabbage. The plant powders and residues were also mixed with ground vegetables. The extracts were given invariably in No. 5 gelatin capsules, and an effort was always made after administering these to get the animals to eat something at once in order to insure the greater part of the dose being swallowed.

FEEDING EXPERIMENTS WITH FRESH MATERIAL

Exper. 1.—Oct. 23, 1917, Belgian Hare 1, male, weighing 1,405 gm. was given 50 gm. of fresh leaves and stems of white snake-root which was gathered at Morris, Ill., from a milksick area on Oct. 20, 1917; some of this was eaten, but not all. Oats and water were available.

Oct. 24: Weight 1,455 gm. More fresh leaves were offered in the afternoon, and some were eaten when they were first placed in the cage; the dried material from the preceding day had not been eaten; oats and water were available.

Oct. 25: Weight 1,340 gm. The rabbit was offered fresh Eupatorium but ate nothing all day. In the evening it sat in the back of the cage, in a more or less humped up position with its head and nose extended; its breathing was short and jerky; and it kept its body swaying from side to side as if it were uncomfortable.

Oct. 26: The rabbit died during the night and the necropsy findings were as follows: The heart was normal in size, light in color, and the vessels were slightly injected. The lungs were somewhat hyperemic. The liver was hyperemic in patches, mottled with clay colored spots, with nutmeg appearance—soft, lobules very prominent, liver not enlarged. The spleen was normal except for the distal end which was dark, due probably to the position in which the animal was lying after death. The kidneys appeared normal. The stomach was full and the intestines and mesenteries were normal. The bladder was distended with urine. No odor of acetone was apparent.

The heart blood and tissues from the liver and spleen were plated in standard agar. All plates were sterile after 24 hours at 37 C.

Exper. 2.—Rabbit 2 was a Belgian hare weighing 1,270 gm. On Oct. 23, 1917, it was given 50 gm. of fresh, green Eupatorium of the same lot as Rabbit 1, but it ate none. Oats and water were given after it refused the weed.

Oct. 24: Weight 1,294 gm. It was again offered 50 gm. of fresh Eupatorium, but none was eaten. After going without food all day it was given oats and water at night.

Oct. 25: Weight 1,310 gm. None of the dry snake-root which was left in the cage from the two preceding days was eaten. No other food was given.

Oct. 26: Weight 1,340 gm. Dried material was offered, but in spite of its apparent hunger it refused to eat. From Oct. 27-30, no effort was made to get it to eat Eupatorium—oats and carrots being fed.

Oct. 29: Weight 1,330 gm. Condition was normal.

Dried snakeroot with a very small amount of oats and carrots was given on Oct. 31, Nov. 1 and 2; almost none of the weed was eaten.

Nov. 3: Weight 1,330 gm. By this time it became apparent that it was useless to endeavor to induce this rabbit to eat the plant by itself, either green or dry, and accordingly 1 gm. of the powdered leaves* from the milksick area was given to it mixed with ground carrots. None was eaten.

Nov. 4: Weight 1,295 gm. A similar mixture of carrots and leaf powder was offered, but none was eaten.

Nov. 5: Weight 1,275 gm. Having failed to get the rabbit to eat any appreciable amount of the carrot mixture, we next tried mixing 0.5 gm. of the leaf powder with chopped cabbage, but with no greater success.

Nov. 6: Weight 1,222 gm. About two-thirds of the cabbage mixture was eaten over night, but when offered a fresh lot, it was refused. Altho losing in weight continually, the rabbit appeared perfectly normal. No other food was given.

Nov. 7: Weight 1,177 gm. Cabbage mixture containing 1 gm. of leaf powder was prepared, but none of it was eaten.

Nov. 8: Inasmuch as the rabbit now refused to eat practically everything which contained the Eupatorium powder, the experiment was discontinued.

* The powdered leaves are somewhat acrid to the human taste, causing the mouth and tongue to smart and burn slightly; the flavor is not unlike that of dried alfalfa or clover leaves and not at all disagreeable. Apparently the rabbit's sense of taste discerns something much more objectionable than the human.

The only deduction that can be drawn from this experiment is that the quantity of snake-root, both green and dried, which the rabbit consumed, was so very small that it was without deleterious effect.

Exper. 3.—Fresh material from the greenhouse, obtained from the roots previously mentioned, was used in this experiment. There were not enough plants from any one locality to permit us to feed that exclusively for any length of time; however, occasionally there was sufficient to furnish material for 2 or 3 days; again, it was often necessary to give a general mixture, so that no attempt was made in this case to draw any inference as to the respective poisonous properties of the plants from the different localities.

Approximately 10 gm. green weight was given at each feeding. Rabbit 8 seemed to be very fond of the green stuff and ate leaves and stems with great relish. The first crop lasted 15 days, during which period 153 gm. were consumed. In the beginning, the rabbit weighed 1,752 gm. and had a temperature of 102.9 F.; after 15 days, its weight was 1,758 gm. and its temperature 103.3 F. In addition to the green *Eupatorium*, it received a supporting ration of carrots and oats. Neither its weight nor temperature at any time varied from the normal enough to suggest anything out of the ordinary, and at the close of this first part of the experiment, the animal appeared to be in as sound condition as ever.

A second crop became available March 11, 1918, but for some unexplainable reason the rabbit refused it unless mixed with other food. About 15 gm. green weight per day were fed. The supply lasted until March 26, fifteen days, during which time 198 gm. were given, of which possibly 150 gm. were eaten.

A third lot was fed with ground carrots, beginning April 4, at the same rate as the last, and continued until April 13. Approximately 85 gm. were eaten at this time.

On April 13, the rabbit refused to eat and sat humped up most of the time; it kept its body swaying from side to side practically all of the time as if uncomfortable, and exhibited a peculiar jerking of the head; at the same time a diarrhea developed. By 7:30 p. m., its breathing had become short and jerky; it seemed to sink into a semi-coma from which it would arouse periodically, throwing its head up and backward. It was still alive the next day, but prostrate, lying on one side with its head thrown back. A partial paralysis of the head and neck had developed, placid in type, but it was still able to raise its head partly. It could not get up on its fore feet, altho the hind parts were still under control. It died at 6 p. m.

The heart showed the right auricle and ventricle dilated as well as the vessels. The lungs were normal. The liver was clay-colored and hemorrhagic. The spleen was normal, while the kidneys were clay-colored, suggesting fatty degeneration; many punctiform hemorrhages were also present. The mesenteries were hyperemic.

Kidney: Glomeruli distinctly hyperemic; capsular space normal size, contains protein granules. Epithelial cells of convoluted tubules are swollen, often filling the entire lumen. The cytoplasm contains many small granules. When stained for fat, all of the epithelial cells of the convoluted tubules are found to stain a deep orange color. The fat droplets are large and more abundant in portions of cells which border basement membrane; similar fatty changes in the loops of Henle, but are not present in collecting tubules.

Liver: Liver cords are poorly defined; cells are large and obliterate the sinusoids. The cytoplasm of liver cells is everywhere granular and many small vacuoles are seen in it. Fat stain reveals many small fat droplets in the liver cells of the peripheral one-third to one-half of liver lobules. No similar changes are noted in cells at center of liver lobules.

Heart: The fibers are slightly more granular than usual and few small vacuoles are seen in them. No other definite change observed. Muscle fibers everywhere contain many small fat droplets.

Poured agar plates were made from heart blood, liver and kidney. These were sterile after 24 hours at 37 C.

The result of this experiment would seem to confirm that of *Exper. 1*, in so far as the ultimate poisonous action of the plant is concerned, although the total green weight consumed, which amounted to some 388 gm., was considerably in excess of that in *Exper. 1*. It should be borne in mind, in this connection, that the greenhouse material was new growth and very succulent, while that fed last fall was mature, ripened tissue from out-of-doors, collected late in October, at blossoming time, and in the latter case, the water content was entirely different, so that on a dry basis there was probably not so much difference as these figures would seem to indicate.

Again, there is the question of the plant being less poisonous at one period of its growth than at another, a consideration which cannot be lost sight of since it is known that with some species such as *Aconitum Napellus* L. or *Monkshood*, the plant is but slightly active when very young, and most active just before flowering, and at minimum activity when the seeds ripen.

Again, the animal may have developed a certain degree of tolerance for the drug, but eventually, the protective mechanism, whatever that may have been, gave way under the repeated administration of the plant.

FEEDING EXPERIMENTS WITH LEAF POWDER

Exper. 4.—The Eupatorium fed in this case was collected at Morris, Ill., Oct. 20, 1917, from a milksick area. The general plan of the experiment was much the same as that of *Exper. 2*, except that no other form of the drug was fed than the leaf powder. Two gm. of the No. 30 powder were given each day with oats, ground carrots or other vegetables. Some of the time all of the dose was eaten, while again, only a portion, so that the amount estimated to have been consumed is, at best, only an approximation.

Rabbit 2-a, weighing 1,760 gm. was given the first feeding on Nov. 7, 1917. It remained normal so far as weight and behavior are concerned until Nov. 26, when it stopped eating and its weight dropped to 1,599 gm., with a temperature of 102.9 F., a little under normal for this animal. It appeared very sick and sat in the characteristic humped-up position as if in pain. The respiration was short, fast and jerky. Toward the middle of the afternoon, it manifested further characteristic symptoms; passing into a semi-conscious condition, it would periodically arouse from this with a jerk; this was repeated 2 or 3 times per minute thruout the afternoon. Less frequently, possibly every 3 minutes, it would change its position, end for end, evidently endeavoring to seek a more comfortable posture. All of this time the head and nose were kept extended. At 9 p. m. it was still alive, but the head and thorax were lying on one side, prostrate, while the hind parts were still upright. When the animal was touched, it was still able to assume an upright position, but was unable to maintain it for any length of time, and at once fell over on its side completely prostrated. No effort was made to regain its former position. The next morning, Nov. 22, it was found dead.

The lungs were normal. The heart vessels were dilated. The liver was not enlarged, but showed marked, light, clay-colored areas; the lobules were very clearly outlined against the hyperemic tissue. The stomach, intestine and mesenteries were distinctly hyperemic. The kidneys appeared normal. The bladder was distended with urine and the stomach was full.

Kidney: The glomerular tufts are small and very markedly hyperemic; the capsular spaces are slightly enlarged and contain protein granules. The intertubular capillaries are greatly distended. All cellular structure of the convoluted tubules is lost, and the cells are so enlarged that no lumen of the tubule is visible. The cytoplasm of the cells is granular, and vacuoles are present, particularly at the periphery of the tubules.

Liver: The liver cords are not well preserved, and the sinusoids are lost. The outlines of the liver cells are indistinct particularly in the peripheral two-thirds. In this part of the lobule the cell nuclei have stained very poorly. The sinusoids contain some red blood cells. The cytoplasm of the liver cells is granular and contains many vacuoles particularly in the peripheral half.

Heart: The outlines of the cells are indistinct. Otherwise the fibers appear normal except for some irregularity in the staining of the nuclei.

Poured agar plates were made from heart blood, liver and kidney, but all were sterile in 24 hours at 37 C.

Our estimate of the amount of leaf powder actually consumed by this rabbit is about 17 gm. Death occurred on the 13th day.

Exper. 5.—Rabbit 3, weighing 1,797 gm., received daily, beginning Nov. 9, two gm. of leaf powder administered as in the preceding case. On Nov. 13, after the feeding had been in progress only 4 days, the animal became sick and failed to eat. All of the symptoms previously mentioned, such as crouching posture, short, jerky breathing, flaccid paralysis of the head and neck appeared, and the rabbit was dead by the morning of Nov. 14.

This is one of the most rapid actions that we have obtained in any of the animals that have received the leaf powder. We estimated the amount of the powder that was actually eaten at 5 gm. Death took place after 5 days.

The heart and lungs were hyperemic, the vessels of the former being dilated. The liver was hyperemic—nutmeg in appearance, soft, but not enlarged. The abdominal viscera and mesenteries were decidedly hyperemic. The spleen was dark. The kidneys were lighter than normal, clay-colored.

Kidney: Glomeruli small, cellular; capsular spaces are large and contain many protein granules indicating albuminuria. Epithelium of convoluted tubules distinctly granular; the outline of the cells is irregular and indefinite and the cells are largely desquamated.

Heart: Fibers of heart muscle contain numerous small vacuoles (fatty degeneration). Nuclei of muscle fibers present a varied appearance; some deep staining and pyknotic while others have distinctly irregular and broken outlines.

Liver: Sinusoids distended with red blood cells and liver cells throughout entire lobule contain many vacuoles. These vacuoles are numerous, each cell containing 6 or 8 visible vacuoles some of which are almost as large as the nucleus of the liver cell.

Spleen: No demonstrable changes.

Poured agar plates were made from the heart blood, liver blood, liver and kidney. All were sterile after 24 hours at 37 C.

Exper. 6.—Rabbit 4, weighing 1,966 gm., was fed 2 gm. of leaf powder daily, mixed with chopped vegetables as in the previous experiments, for a period of 8 days. On the 8th day it ate practically nothing and died on the 9th day, Nov. 18.

The symptoms manifested on the last day were in all respects the same as those already described, except that there was a slight diarrhea.

During the 9 days, the rabbit consumed approximately 10.5 gm. of the leaf powder.

The vessels of the heart were dilated. The lungs were somewhat hyperemic. The liver presented the typical nutmeg aspect and was hyperemic, mottled with lighter areas. The stomach was full. The mesenteries were hyperemic and the kidneys as well as the spleen were normal.

Kidney: The glomerular tufts are contracted and hyperemic; the capsular spaces are enlarged and contain many protein granules. The lumen of the convoluted tubules appears to be entirely closed due to the greatly enlarged and irregular epithelial cells. The outlines of the cells are scarcely visible, and the cytoplasm is granular and contains many small granules. The intertubular capillaries are distended with red blood cells.

Liver: The liver cords are poorly defined and the sinusoids are distended with red blood cells. The liver cells are somewhat larger than normal and the cytoplasm contains many large and small vacuoles. The nuclei appear to stain equally well thruout the lobule.

Heart: The muscle fibers contain numerous small vacuoles.

Spleen: The spleen appears to be normal.

Poured agar plates were made from the heart blood, liver and kidney, but all were sterile after 24 hours at 37 C.

Exper. 7.—Rabbit 5, a pregnant female, weighed 2,222 gm. She had a ravenous appetite, doubtless due to her condition, and on the 1st day, Nov. 20, she ate 3 gm. of leaf powder mixed with oats. By the next day she seemed to have developed a dislike for the material and upset the dish repeatedly which contained the oats and snake-root. Finally she ate, possibly, one-half of the dose. On the 3rd day, she refused all food, and by 5 p. m. she was very sick and hardly able to sit up. She appeared to be hungry and made several attempts to eat, but failed almost completely. By evening, she was prostrate, unable to sit up and died during the night.

The thoracic viscera were normal. The liver was uniformly hyperemic and presented the nutmeg aspect. The left kidney was somewhat enlarged, the right, normal; both were slightly hyperemic and yellow gray in color. The spleen was normal. The mesenteries and intestines were normal.

Kidney: The glomerular tufts are somewhat contracted and are extremely hyperemic; the capsular spaces are enlarged and contain but little protein material. The epithelial cells of the convoluted tubules are enlarged so that they fill the lumen of the tubules almost completely. The outlines of the cells are indistinct and the cytoplasm is granular and contains many small vacuoles. The intertubular capillaries are distended with red blood cells.

Liver: The liver cords are fairly well defined; the sinusoids contain some red blood cells. The liver cells are enlarged and the nuclei in the peripheral third stain poorly. The cytoplasm contains many vacuoles especially at the margin of the lobule.

Heart: The muscle fibers appear normal.

Spleen: The spleen appears normal.

Poured agar plates were made from the heart blood, liver, spleen and kidney, but all were sterile after 24 hours at 37 C.

A very liberal estimate of the leaf powder eaten in this case would be 4 gm. and death followed in 3 days. This is the most rapid action that was obtained in any of the leaf powder experiments.

Exper. 8.—The picture which this experiment presents is essentially the same as that given by the preceding.

Rabbit 6, weighing 1,645 gm., was fed the leaf powder with chopped vegetables, and on the 3rd day it seemed rather listless and ate but little. Here, too, as in practically all of the cases previously described, the animal seemed to be very hungry and came to its food as if possessed with a ravenous appetite, but after the first mouthful, all desire for food seemed to vanish. A slight diarrhea developed. On the next day, Nov. 13, it appeared better and ate a little. By Nov. 14, it manifested typical symptoms of Eupatorium poisoning and was dead by the morning of the next day, Nov. 15.

The heart vessels were dilated. The lungs were normal. The stomach was gorged. All of the abdominal viscera were hyperemic, particularly the mesenteries. The liver presented the characteristic nutmeg appearance and was uniformly hyperemic; not enlarged. The kidneys and spleen were normal. The bladder was distended with urine.

Kidney: The glomerular tufts are very much contracted and hyperemic. The capsular spaces are enlarged and contain many protein granules. The epithelial cells of the convoluted tubules are but slightly swollen and the lumen of the tubules is rather well defined.

Liver: The liver cords are fairly well preserved; the sinusoids are distended with red blood cells. The cytoplasm of the liver cells is very granular and contains a few vacuoles.

Heart: The muscle fibers contain many very small vacuoles, but are otherwise normal.

Poured agar plates were made from heart blood, liver and kidney, but all were sterile after 24 hours at 37 C.

This rabbit did not consume to exceed 4 gm. of the leaf powder and died 6 days from the time the feeding began.

It should be mentioned, in passing, that all of the animals were given plain oats, in quantity sufficient to maintain their normal weight, provided they ate them, but only after ample opportunity, 6-8 hours, had been offered them to eat the powder-vegetable mixture. It was clearly apparent in every case that there was something objectionable to the rabbit about the powder, for as a rule they ate it very reluctantly.

Exper. 8.—Control.—In order to make certain that our experimental animals were receiving the proper amount of food and the necessary combinations to maintain their normal weight under perfectly normal conditions, Rabbit 7, weighing 1,794 gm., was fed as a control. It received the same ration as the other rabbits, except for the fact that its food contained none of the Eupatorium. On Nov. 22, which terminated the experimental period for all of the rabbits that were fed leaf powder, this control weighed 1,775 gm., or 15 gm. less than at the beginning, a difference which falls easily within the normal daily range.

In view of the facts, that this animal which received no leaf powder, remained perfectly well and maintained its weight practically constant throughout the duration of the experiment, and whereas all 5 which ate the Eupatorium died in from 3-13 days with uniformly typical symptoms, the conclusion seems warranted that the latter came to their death as a result of Eupatorium poisoning.

FEEDING EXPERIMENTS WITH ALCOHOLIC EXTRACTS OF EUPATORIUM URTICAEFOLIUM LEAF POWDER

Exper. 10.—The solid alcoholic (95%) extract, prepared as described elsewhere, was fed to Rabbit 14 at the rate of one capsule per day for a period of 9 days. Each capsule contained an average of 161.65 mg. of the extract, which corresponds to approximately 2.8 gm. of the leaf powder, or for the whole time, the animal received extract equivalent to about 25 gm. of leaf powder.

The feeding was begun Feb. 2, 1918, on which date the rabbit weighed 2,055 gm., temperature 103.8 F. It remained normal until Feb. 13 when it stopped eating; weight 2,033 gm., temperature 102.8. It was very sick on the 15th, with a temperature a little under normal, 100.5. The body was relaxed, the head lopped to one side, placid paralysis of the head and neck. It died before the morning of the next day, Nov. 16.

The heart was enlarged, dilated and hyperemic. The lungs were somewhat hyperemic. The liver was hyperemic and cream-colored in patches. The kidneys were lighter in color than normal with many punctiform hemorrhagic spots visible through the capsule; these were suggestive of infarcts. The intestines and mesenteries were hyperemic.

Kidney: The glomerular tufts are somewhat shrunken and hyperemic. The capsular spaces are about normal in size and contain many protein granules. The cells of the convoluted tubules are greatly swollen, indistinct and irregular in outline and practically close the lumen of the tubule. The cytoplasm is very granular and contains many small vacuoles.

Liver: The liver cords are fairly well preserved at the center of the lobule but are lost in the peripheral half. The nuclei in the liver cells of the peripheral half of the lobule do not stain at all or only feebly. The sinusoids are also lost in this part of the lobule. The cytoplasm of the enlarged liver cells is granular and contains many small vacuoles.

Heart: The fibers are poorly outlined and are slightly more granular than normal. The cytoplasm contains many very fine vacuoles.

Poured agar plates were made from heart blood, liver and kidney, but all were sterile after 24 hours at 37 C.

Exper. 11.—As in the preceding experiment, the solid alcoholic extract, prepared from leaves gathered near Morris, Ill., was fed to Rabbit 15, beginning Feb. 17, 1918. At that time, the animal weighed 1,512 gm., and its temperature was 103.3 F. One No. 5 capsule was given each day for 18 days, an amount corresponding approximately to 28 gm. of the dry leaf powder. The rabbit appeared normal until the 18th day when it stopped eating. Weight 1,476 gm., temperature 102 F. By the next day, March 8, it was very weak and could be pushed over easily, but was neither prostrated nor paralyzed yet. It died on the next day with typical symptoms as previously noted, 20 days after the feeding was begun.

Punctiform hemorrhages were present in the thymus gland. Lungs were normal. The heart had a pale, light, cooked appearance. The stomach was full. The kidneys were lighter colored than normal—claylike. The liver was hyperemic with nutmeg appearance. The spleen was normal. The bladder was not entirely filled; urine neutral to litmus.

Kidney: The glomerular tufts are greatly contracted and hyperemic; the capsular spaces are not enlarged, but contain protein granules. The epithelial cells of the convoluted tubules are swollen, irregular and nearly close the lumen of the tubule; the cytoplasm is granular and contains many small vacuoles. The intertubular capillaries are distended with red blood cells.

Liver: The liver cords are well preserved and the sinusoids are distinct but distended with red blood cells. The nuclei stain uniformly thruout the lobule; the cytoplasm of the liver cells contains many vacuoles, particularly those of the peripheral third.

Heart: The muscle fibers contain many small vacuoles.

Poured agar plates from heart blood, liver and kidney were sterile after 24 hours at 37 C.

The results of the last two experiments indicate very clearly that the leaves of *E. urticaefolium* contain an alcohol soluble active principle which is capable of causing death when fed to rabbits.

Exper. 12.—The alcoholic extract fed in this instance was prepared from the dried stems and leaves of material received from Massachusetts; in fact, it was mostly brown, frosted stems with very little leaf tissue. Rabbit 9, weighing 1,380 gm., temperature 103.7 F. on Jan. 23, 1918, received this extract without ill effects until the equivalent of 60 gm. of the stem-leaf powder had been taken. At this point we were forced to discontinue the experiment since the rabbit had developed an infected jaw several days previously, as a result of which it died on Feb. 9, eighteen days after the experiment was begun. Up to the time of death, the animal had manifested none of the symptoms of *Eupatorium* poisoning, and the necropsy failed to show anything characteristic. The bacteriologic findings indicated that death was probably due to a general septicemia.

Up to the time this jaw infection occurred, stem extract, corresponding to approximately 30 gm. of the dry powder, had been given with negative results, and at the time of death the equivalent of 60 gm. had been consumed.

Taking into consideration the fact that no symptoms of poisoning were manifested at any time during life, and that the postmortem examination showed nothing characteristic, we are reasonably safe in concluding that the extract from the Massachusetts stems contained very little, if anything, that was poisonous for this rabbit in the quantity which was taken, representing more than 2 times the amount of the Morris leaf powder that was necessary to kill.

It should be remembered in connection with this material that it was largely dried stems, and subsequent tests have shown that stem extracts, even from Morris plants, are not poisonous for rabbits, at least when 3 times the lethal dose for leaf extract is administered.

Exper. 13.—Up to this point, our experiments have dealt only with rabbits; it seemed worth while to see what effect our different preparations might have on guinea-pigs. Accordingly, Pig 1 was fed on the alcoholic extract, similar to that given to the rabbit in the preceding experiment, that is, from Massachusetts stems. The dosing began Jan. 25, 1918, and was discontinued Feb. 28, after 33 days, during which period the animal had taken extract corresponding to 110 gm. of the dry powder, an amount equivalent to almost one-fourth of its body weight. At no time was there any indication of any abnormal condition, and accordingly it appears that the alcoholic extract of Massachusetts stems is equally as harmless for guinea-pigs as for rabbits.

FEEDING EXPERIMENTS WITH PHYSIOLOGIC SALT SOLUTION EXTRACTS OF EUPATORIUM LEAVES

Exper. 14.—The large amount of soluble material which the leaves yield to extraction with physiologic salt solution complicates considerably the problem of feeding the extract to laboratory animals. In the first place, as mentioned before, it is often impossible to get a rabbit to take more than one capsule at a time. This is particularly true when the material administered contains a bitter principle, such as is present in our extracts. In the second place, when the dosage has to be strung out over a long period, for the reason just mentioned, and it is necessary when such bulk has to be given, questions of tolerance and elimination always enter in as complicating factors.

Recognizing these as possible sources of error in drawing conclusions, but having at our disposal at that time no method of concentrating the active principle from the salt solution extract, we, nevertheless, fed Rabbit 7 with the solid extract corresponding to 24 gm. of leaves. This was administered in 37 doses over a period of 36 days. The experiment was discontinued at this point, owing to the fact that the animal had developed snuffles from which it died 2 days later.

The extract seemed to have a rather drastic action on the intestine, due possibly to the sodium chlorid, for thruout the feeding there was more or less diarrhea. No symptoms of *Eupatorium* poisoning were observed at any time, and the necropsy findings failed to show anything at all characteristic. It is to be regretted that the rabbit died from a secondary cause after being carried for nearly a month and a half, during which time it had received daily doses of the water extract.

So far as the results of this experiment go, it appears that the salt solution extracts nothing from the leaves in the way of an active principle which is injurious for rabbits.

FEEDING EXPERIMENTS WITH THE ETHER-CHLOROFORM-AMMONIA EXTRACT
OF EUPATORIUM LEAF POWDER

The most convincing and uniform results that we have obtained in all of our work have been secured with the ether-chloroform-ammonia extract of leaves from the Morris plants, gathered at blossoming time. Among the several reasons that may account for this, the following may be mentioned:

1. The poisonous principle may be more readily soluble in this solvent complex than in the other solvents employed.

2. This solvent removes less inactive material to dilute the poison than the others.

3. The bulk of the extract being so much less, it has enabled us to give the active principle in larger and more concentrated doses in a shorter time.

4. The solvent may have converted the poison into a form more readily absorbed by the animal cells, that is, into a free state.

In practically every case, it was possible to put the entire solid extract from 15 gm. of leaf powder into 3, and, at most, 4 No. 5 capsules, and unless otherwise stated, this has been the regular procedure adopted thruout the next few experiments. One capsule was given every 24 hours until the extract from 15 gm. of leaf powder had been given.

Exper. 15.—Rabbit 13, weighing 2,144 gm., temperature 102.8 F., received one capsule per day for 3 days, beginning Feb. 14, 1918. On the 3rd day, considerable difficulty was met with in getting it to take the dose. We have observed this same thing since then in a number of cases, namely, that when the animals have been sickened by the first two capsules they invariably object to taking the third. It is easily conceivable that they may have learned to associate the taste of the medicine with their condition.

This rabbit ate nothing the 4th day; it appeared weak and kept the body swaying from side to side. Its weight was a little under normal, 2,111 gm., and its temperature had fallen to 100.2. On the next day, Feb. 18, it developed a flaccid paralysis of the front legs and muscles of the neck, so that its head remained in whatever position it was placed. The hind parts were only partially paralyzed, but the animal was not able to stand or move about. Toward noon it became prostrate with its head thrown back, respiration rapid and shallow. A distinct sweetish, chloroform odor could be detected around the body. It was dead on the morning of the 19th, five days after the first dose was taken.

Sweetish, chloroform odor very pronounced. The lungs were normal. The heart was dilated and hyperemic. The liver was hyperemic with typical nutmeg appearance. The spleen was normal. The kidneys showed distinct punctiform hemorrhages. The bladder and stomach were filled. The brain and cord appeared normal.

Kidney: The glomerular tufts are somewhat contracted and hyperemic. The capsular space is enlarged and contains many protein granules. The epithelial cells of the convoluted tubules are swollen so as nearly to close the lumen. The outline of the cells is indistinct and many small vacuoles are present; the inter-tubular capillaries are prominent.

Liver: The sinusoids are distended with blood; the liver cords are not well defined. The liver cells are everywhere granular and contain many vacuoles of considerable size.

Heart: The muscle cells are poorly defined and the nuclei do not stain uniformly; the cytoplasm contains extremely fine vacuoles in great numbers.

Agar plates were prepared from heart blood, liver and kidney, but all were sterile after 24 hours at 37 C.

Exper. 16.—Rabbit 16, weighing 1,202 gm., temperature 103.7 F., was given the extract from 15 gm. leaf powder, distributed over 3 days, beginning Feb. 28, 1918. Its condition remained normal until the 4th day when its temperature dropped to 100.9 and its weight to 1,150 gm. It ate almost nothing and seemed very sick and weak; sat with its eyes half closed, ears drooped, listless and with the body humped up. It kept moving about and changing its position as if trying to find one in which it would be more comfortable. It was dead on the morning of March 4, five days after the first capsule was taken.

Chloroform odor of the body very pronounced, particularly when the skin was removed. The lungs were normal. The heart was not enlarged, but hyperemic. The blood vessels of the skin seemed to be distended more than usual. The liver was hyperemic with characteristic nutmeg appearance, though not as light in color as sometimes. The kidneys were lighter in color than normal, and one showed punctiform hemorrhages. The spleen was normal. The stomach was full and hard as seemed to be the case almost invariably. The mesenteries were hyperemic. The bladder was distended with urine which was neutral to litmus.

Kidney: The glomerular tufts are small and somewhat contracted but rather definitely hyperemic; the slightly enlarged capsular space contains protein granules; the capillaries between the convoluted tubules are more prominent than usual. The epithelial cells of the convoluted tubules are increased in size, filling the lumen nearly completely. The cytoplasm is granular; the outlines of the cells indefinite; numerous small vacuoles occur in the cytoplasm.

Liver: The cells at center of liver lobule appear practically normal; at periphery of lobules the nuclear stain is lost completely in peripheral third of some lobules; the cytoplasm of the cells is granular and their outlines are indefinite. In other lobules the changes are less marked but are always more prominent at periphery.

Heart: Only a few small vacuoles are seen in the muscle fibers.

Poured agar plates were made from heart blood, liver and kidney, but all were sterile after 24 hours at 37 C.

Exper. 16.—Thus far, all of the extracts employed in this series as well as those in the others which had proved fatal, had been prepared from leaves obtained from a "milk-sick" area near Morris, Ill. It will be recalled that material was also collected from a second tract adjoining the former, except for an intervening wagon road, and that altho it was reported that cattle did not sicken from eating the snake-root which grew there, the writer is of the opinion that this phenomenon was due to the fact that the stock found other forage here and did not eat the snake-root, rather than that the weed was not poisonous on this side of the road. Be that as it way, we decided to put the question to a test, and accordingly extracted 15 gm. of leaf powder from the disease-free area by the ether-chloroform-ammonia method. This was fed to Rabbit 18, weighing 1,046 gm., temperature 102.5 F., in three capsules as previously described. The animal remained normal until noon of the 4th day, March 7, when it became very weak, and by 2 p. m. it was hardly able to hold up its head. Apparently, the well recognized, flaccid paralysis was coming on, for its head was now lopped to the left side; by 2:30 p. m. it was prostrate in the cage, nose and head extended, but still able to right itself temporarily, when disturbed, after much exertion. It was dead by 3:30 p. m. There was almost no loss of weight and no subnormal temperature during the 4 days of the experiment.

The lungs were normal. The heart was hyperemic. The liver was hemorrhagic and did not have usual nutmeg appearance, but mottled with clay colored patches. The kidneys seemed normal. The mesenteries were hypermic. The bladder was distended with urine. The brain and cord were normal. A distinct sweetish, chloroform odor was present about the body.

Kidney: The glomerular tufts are greatly contracted and hyperemic; the capsular spaces are somewhat enlarged and contain many protein granules. The epithelial cells of the convoluted tubules are somewhat swollen and irregular, but not to such an extent as to close the lumen of the tubule. The cytoplasm of the cells is granular. The intertubular capillaries are distended with red blood cells.

Liver: The liver cords are well preserved and the sinusoids are clearly defined. The liver cells appear of normal size thruout the lobule and the cytoplasm contains numerous vacuoles.

Heart: The muscle fibers appear normal.

Poured agar plates from the heart blood, liver and kidney were sterile after 24 hours at 37 C.

The results of this experiment make it quite clear and unmistakable that the *Eupatorium* even from a "disease free" area contains an active principle poisonous for rabbits and in all probability likewise poisonous for cattle should they eat the plants in sufficient quantity.

SHAKING METHOD OF EXTRACTION VS. INFUSION METHOD

Exper. 18.—In order to ascertain whether it was actually necessary to "shake out" the poisonous principle by vigorous agitation for 2 hours, as was our practice, or whether the same result could be accomplished by simple maceration for the same length of time, 15 gm. of leaf powder, previously shown to be poisonous, were covered with the ether-chloroform-ammonia mixture and allowed to stand for 2 hours with only occasional agitation. At the end of this time the fluid was decanted and evaporated as usual. The solid extract was fed to Rabbit 11 in 5 capsules without harmful results.

In the light of later experiments, dealing with the apparently harmless action of small consecutive doses, it may be that the negative results obtained here should be attributed to the method of administration rather than to the failure of the maceration process to extract the poison. At any-rate, the experiment served as a most excellent check on any possible poisonous chemical complex which might be formed by the solvents themselves. That no such complex is formed, has been demonstrated repeatedly in experiments cited later, in which the same solvents have been employed and in which the extracts obtained gave negative results.

The rabbit, used for the first part of this experiment, was rested for 15 days and then given the solidextract from 15 gm. of leaf powder by shaking the latter vigorously for 2 hours with the solvents enumerated. This was administered in 3 doses on 3 consecutive days. On the 2nd day, March 16, the animal seemed sick, and on the 3rd day, it ate almost nothing. Its weight dropped from 2,080 gm. to 1,900 gm. and its temperature from 103 to 100.2 F. By the morning of the 4th day, its head and neck exhibited flaccid paralysis, and by noon the whole body was paralyzed and the animal was unable to right itself when pushed over. It died at 2:30 p. m. on the 4th day, March 18.

Distinct sweetish, chloroform odor to the body when the skin was removed. The heart was dilated, and had a light, cooked appearance. The lungs were normal. The liver was hyperemic with usual nutmeg aspect. Both kidneys were thickly studded with punctiform hemorrhages. The mesenteries were decidedly hyperemic. The spleen was normal. The stomach was full. The bladder was distended with urine which was neutral to litmus.

Kidney: The glomerular tufts are somewhat contracted and hyperemic. The capsular spaces are slightly enlarged and contain protein granules. The epithelial cells of the convoluted tubules are swollen so that the lumen is nearly closed. The cells are irregular in outline; the cytoplasm is granular and contains numerous small vacuoles. The intertubular capillaries are distended with red blood cells.

Liver: The liver cords are rather poorly preserved and the sinusoids contain many red blood cells. The liver cells are somewhat larger than normal and the nuclei stain rather irregularly throughout the lobule. The cytoplasm is granular and contains many small vacuoles.

Heart: The fibers of the heart muscle contain many very fine vacuoles.

Poured agar plates from heart blood, liver and kidney were sterile after 24 hours at 37 C.

The result of the latter part of this experiment indicates at least two things:

1. That the extract obtained by the "shaking out" method was quite poisonous for the rabbit.

2. That the rabbit did not possess a natural immunity which might explain the failure of the maceration extract to kill.

The rather quick action of the shaken extract, manifesting itself on the 2nd day, might be explained on the ground of cumulative action resulting from the material taken 15 days previously, or by assuming that the protective mechanism of the organism had been so weakened by the earlier treatment, that a given amount of the poison was able to produce more injury at this later date than would be the case in a normal rabbit. As shown by the next experiment, we have little reason for believing that anaphylaxis plays any significant rôle in the action of the poison, and therefore the explanation offered in the foregoing seems the more tenable one.

ANAPHYLAXIS

Exper. 19.—In order to determine what part, if any, anaphylaxis played in the behavior of the poison, Rabbit 20 was given one of 4 capsules prepared from the extract of 15 gm. of leaf powder on March 22, 1918. No more were given until April 5 and 6, fourteen days from the time the first was taken, on which dates 2 of the remaining 3 doses were given; the fourth, which contained about one-eighth of the total extract, was not used. The day after the first one was fed, that is, March 22, the rabbit seemed a bit sick, but recovered and appeared perfectly normal with good appetite and increased in weight until April 7, the day following the third capsule. It ate nothing and by 4:30 p. m. paralysis of the hind legs had developed while the front legs, head and neck remained active, in which respect this case differed from all previous ones. As it lay in the cage its hind legs were sprawled to either side and when it endeavored to move about, it dragged the body by the front legs, apparently unable to make any use of the hind ones. It was still alive on the 8th, but prostrate and unable to get up. It died at 10 a. m.

The heart was enlarged, dilated and hyperemic. The thymus gland was enlarged. The lungs were normal. The stomach and bladder were full. The liver was mottled with light and dark patches, hyperemic with nutmeg appearance. The kidneys were light colored with many very fine punctiform hemorrhages showing thru the capsule. The mesenteries and intestine were hyperemic. The cutaneous vessels were dilated. The brain and cord appeared normal.

Kidney: Glomerular tufts small, shrunken and hyperemic; the enlarged capsular spaces contain protein granules; cells of the convoluted tubules are swollen, granular and their outline is irregular. The intertubular capillaries are prominent.

Liver: Liver cords are fairly well preserved; the sinusoids are prominent. The cytoplasm of the liver cells is slightly more granular than usual, and small vacuoles are seen especially in cells in peripheral half of liver lobule.

Heart: Muscle fibers are fairly normal in appearance, containing few small vacuoles.

Poured agar plates made from heart blood, liver and kidney were sterile after 24 hours at 37 C.

There is nothing about the results of this experiment to indicate that there was any anaphylaxis; there was nothing in the deportment of the rabbit following the first dose after the sensitizing dose to suggest shock, and the animal developed the usual symptoms within the regular interval and died in the customary manner. It does appear, however, that this rabbit was more susceptible to the poison, either naturally or because of the effect of the first capsule, for whereas 3 capsules given in succession were ordinarily necessary to kill, here less than 2 proved fatal. It is altogether possible that the active principle exerts a cumulative effect, and that the protective mechanism of the animal was weakened by the first dose, and that in the interim of 14 days, it had not recovered sufficiently to withstand the two following doses, and therefore, reacted toward these just as it would toward any three capsules given in succession.

The rabbit weighed 1,616 gm., temperature 102.4 F. at the beginning of the experiment and 1,736 gm., temperature 101.9, two days before it died.

DOSAGE AND CUMULATIVE ACTION

Exper. 20.—As a rule, in all of our feeding experiments with the ether-chloroform-ammonia extract, the material had been administered in either 3 or 4 doses 24 hours apart. This number was selected because of the fact that the solid extract from 15 gm. of leaf powder could be accommodated in three No. 5 capsules very easily. They were given 24 hours apart because of the difficulty we experienced in getting the rabbits to take them more frequently, and with this administration, we invariably secured positive action.

The preceding experiments having demonstrated beyond all reasonable doubt that this extract contains a substance poisonous for rabbits, we next turned our attention to the effect of administering the lethal dose at different rates.

In the experiments just reported, and more especially, perhaps, in those with the fresh greenhouse *Eupatorium* and with the alcoholic extracts, there was some reason to suspect that cumulative action was playing a part. One might very naturally expect the same fatal result to follow the administration of the lethal dose of a poison, if it were a cumulative poison, whether given in one large dose or in several smaller ones at frequent intervals, providing, of course, that the amount of the active ingredient ingested each day was in excess of that excreted by the organism. This, however, does not necessarily follow, for it is an observed fact that a large dose of lead acetate, for example, may be taken at one time without any noticeable effect, whereas small amounts taken daily, would, in all probability, result in serious poisoning. According to Rosenau¹⁰ "the reason for this is that where one large dose is taken only a small quantity is absorbed; the rest is swept through the intestines, but when small quantities are taken at frequent intervals practically all is absorbed and the metal accumulates in the tissue, poisoning especially the delicate nervous structures."

In order to determine the behavior of *Eupatorium* poison when taken in one large dose, Rabbit 19 was given the extract from 15 gm. of leaf powder at one feeding on March 20, 1918. The 2nd day it appeared rather indisposed, ate nothing and sat humped up—symptoms usually exhibited on the 3rd and 4th day by animals which have received split doses. The weight dropped from 1,660 to 1,622 gm. and the temperature from 103.2 to 102.2 F., differences which might easily fall within the individual variation. The 3rd day the rabbit seemed normal and showed no ill effects thereafter. It gained in weight steadily and is still under observation, now (5/29/18) weighing 2,185 gm. Aside from the slight indisposition exhibited by the rabbits on the day after receiving the drug, the one large dose taken at one time produced no further reaction.

Exper. 21.—By way of confirming the results obtained in the last experiment, a second animal, Rabbit 12, was given the lethal dose at one time on May 15. It appeared perfectly normal the following day, except for a slight loss in weight, and since then has never shown any indication of *Eupatorium* poisoning.

The results of the last two experiments seem to point to an action similar to that described in connection with lead poisoning, for manifestly there is not the same quantity of poison absorbed from one large dose as from the 3 or 4 smaller ones.

¹⁰ Preventive Medicine and Hygiene, 3d Ed., 1917, p. 1048.

Exper. 22.—With a view to ascertaining how rapidly the active constituent was excreted and if any cumulative effect could be observed where the lethal dose was distributed over more than 3 or 4 days, we fed Rabbit 19 the extract from 15 gm. leaf powder, divided into 8 portions, over a period of as many days.

So far as we were able to detect, both by eye and with scale and thermometer, the animal remained normal throughout the duration of the experiment. Apparently, in this case, the poison in the small recurring doses was excreted as rapidly as it was ingested, and none of it was retained to produce subsequent injury to the tissue, whereas in the larger doses—3 or 4 capsules—the rate of elimination is not sufficiently rapid to prevent the absorption of injurious quantities of the active principle.

STEMS

Exper 23.—While some plants are poisonous in all their parts, the various structures differing only in the degree of toxicity, there are also those in which the active constituent appears to be concentrated in a definite part as in the seeds of *Agrostemma Githago*, L. (corn cockle), in the root of *Sium latifolium*, L. (water parsnip), and in the leaves of *Conium maculatum*, L. (hemlock). This is particularly true of the last mentioned in the early summer.

Cattle browsing on snake-root would be very apt to ingest more or less stem tissue along with the leaves, and therefore we prepared an extract from the stem powder of plants collected at Morris, Ill. The amount of soluble matter which 15 gm. of stems yielded to the solvent complex was less than one-third of that obtained from the leaves, and was easily accommodated in one No. 5 capsule.

The extract from 60 gm. of stem powder was fed to Rabbit 22 in 4 doses, the equivalent in extract of four 15 gm. portions of stems, on 4 different days beginning March 4. The first 3 capsules were taken at intervals of 24 hours without any perceptible change in the rabbit's condition, and 4 days from the time the third was given, the fourth was administered, but without harmful effect. The rabbit ate well and continued to gain in weight.

It appears from this that the stems contain little, if any, of the active principle compared with the leaves, since the extract corresponding to 4 times the lethal dose of leaves was fed without visible injury.

Roots

Exper. 24.—A bitter principle has been noted as occurring in the rhizomes and rootlets of *E. purpureum* and *E. aromaticum*. As to the presence of any active principle in the roots of *E. urticaefolium*, we have no knowledge.

It is easily conceivable how cattle might be poisoned in the spring by consuming whole plants, assuming the roots to be poisonous also, for at this season the ground is apt to be soft, and little effort is required to pull up the tender shoots, roots and all.

We have endeavored to ascertain the presence of an active principle in the rhizomes and rootlets of our species by feeding Rabbit 19 with the extract from 60 gm. of root powder, administered as in the preceding experiment. Here also the bulk of the extract was much less than that from the leaves. Altho the extract corresponding to 4 times the lethal dose of leaf extract was fed, there was not the slightest indication of any injurious action.

From this it appears that the underground structures are deficient in any active constituent soluble in ether-chloroform-ammonia when tested out on rabbits.

Exper. 25.—It will be recalled that in *Exper. 13* a guinea-pig was fed with the alcoholic extract from Massachusetts stems and leaves, but without harmful results.

In the present case, Guinea-pig 2 was given the ether-chloroform-ammonia extract from 45 gm. of leaf powder in 9 capsules at 24-hour intervals. The pig remained perfectly well, had a good appetite and gained in weight thruout the experiment. It should be noted here that the animal received 3 times the amount necessary to kill a rabbit and that the dosing was carried out at the same rate as proved fatal for rabbits.

Exper. 26.—A leaf extract prepared from material received from Minnesota, Oct. 17, 1917, was fed in this experiment. The quantity of solid extract obtained from a given weight of this powder was practically double that from a similar weight of Morris material. The reason for this was not apparent at the time, but as will be seen from the results of the experiment, we were obviously dealing with a new set of conditions. In all, Rabbit 17 took the extract from 45 gm. of leaf powder; that from the first 15 gm. was given in 6 capsules at intervals of 24 hours, but with no effect. In the interim that followed, *Exper. 22* was completed, the results of which indicated that small doses over a long period were not effective. Since the extract in the present case had been fed in 6 portions, failure to obtain positive results may have been due to a similar cause, namely, the rapid elimination of the poison. Accordingly, the experiment was repeated, and the same rabbit was given the extract from 15 gm. of Minnesota leaf powder in 3 doses on April 29, 30 and May 1, and again, that from an additional 15 gm. on May 2, 3 and 4, but without effect. Altogether it had received 3 times the amount of the Morris extract required to kill.

Several factors may have contributed to the failure to obtain positive results:

1. The plants may have belonged to a different species of *Eupatorium* than *E. urticaefolium*.
2. They may have failed to develop a poisonous constituent under Minnesota conditions.
3. The Minnesota plants were just coming into bloom, whereas those from Morris, Ill., were just about through that stage—a point to be taken into consideration if we assume for the present that some relation exists, within very narrow limits, between the appearance of the poison and the time of blossoming.
4. The leaf powder may have deteriorated although kept under identically the same conditions as that from Morris.
5. The rabbit may have been naturally immune.

THE SOLVENT COMPLEX

Exper. 27.—While there was little ground, either chemical or physiologic, for believing the fatal results obtained in the foregoing experiments were due to a poisonous compound formed from the solvents themselves, we have, nevertheless, checked this point still further by extracting 45 gm. of *Eschlepias verticillata* (whorled milkweed) with the ether-chloroform-ammonia mixture just as was done with the leaf powder. The residue was fed in 3 doses at 24-hour intervals to Rabbit 21 with no deleterious effect whatever.

This result, together with those of similar experiments in which the same solvents were employed as in *Expers. 20, 21, 22, 23 and 24*, make it obvious that the solvent complex plays no part in the action of the extracts.

FEEDING EXPERIMENT WITH RABBIT TISSUES

Exper. 28.—As mentioned elsewhere, the literature on milksickness makes frequent reference to the danger from using the meat and milk of animals affected with the disease. Experimental evidence has been produced which seems to indicate that such statements are not wholly without foundation.

An opportunity of carrying out work along this line was afforded us with the carcasses of rabbits that died in the laboratory from *Eupatorium* poisoning. On Feb. 16, 1918, a half grown cat, weighing 2,505 gm. was fed the heart, lungs, liver, kidneys and spleen of Rabbit 14 which died from the alcoholic extract of leaf powder. On the two following days it ate 470 gm. of the meat. On the 20th, it was given the heart, lungs, liver, kidneys and spleen of Rabbit 13, which died from the ether-chloroform-ammonia extract. On the three following days it ate 672 gm. of meat from this animal. No injurious effects were noted. On March 4 it received the heart, lungs, liver, kidneys and spleen of Rabbit 16 (*E. C. A.* extract) and on the 2 following days 318 gm. of the meat. On March 8, it was

given the viscera enumerated above and 95 gm. of meat from Rabbit 18 (E. C. A. extract); on the next day, 175 gm. meat from Rabbit 18 and the viscera of Rabbit 15 (alc. extr.); on the two following days it ate 438 gm. of meat from Rabbit 15. On March 19, it was given the viscera of Rabbit 11 (E. C. A. extr.) and on the 4 following days a total of 680 gm. of the meat. On April 9, it ate the viscera together with 100 gm. of meat from Rabbit 20 (E. C. A. extract); during the next 2 days, it received 362 gm. of the meat. Once more, on April 15, it was given the heart, lungs, liver, kidneys and spleen and 150 gm. of the meat from Rabbit 8 (Greenhouse Eupatorium); on the next 2 days it consumed the remainder of the meat which amounted to 387 gm.

While being fed on the rabbit tissues, the cat received nothing else but pasteurized, sweet milk, and between times, it was given fresh raw beef and sweet milk. Altogether, the animal consumed the viscera indicated and the muscle tissue of 8 rabbits—the meat alone amounting to 3,970 gm. or more than one and one-half times its own body weight.

The cat was playful all of the time and remained perfectly healthy and normal thruout the experiment. It increased in weight from 2,505 gm. on Feb. 2, to 3,066 gm. on April 17—a net gain of 561 gm. in 59 days.

FEEDING EXPERIMENTS WITH EXHAUSTED RESIDUES

In order to determine whether the leaf powder had yielded its poisonous principle to the different solvents employed, and as a further check on the toxicity of the resulting extract, the leaf residues from the different extractions were fed to rabbits.

RESIDUE FROM 95% ALCOHOL EXTRACTION

Exper. 29.—Rabbit 12 was fed the dried residue from 30 gm. of extracted leaf powder, mixed with chopped carrots, at the rate of 2 gm. per day for 15 days. The animal ate the residue readily and with apparent indifference to its presence in the food.

There was no indication of any injurious effect at any time, altho the total quantity consumed represented more than two lethal doses before extraction for an average rabbit. The extract from this material was fed to Rabbit 14 (*Exper. 10*) and resulted fatally.

It appears from this that the 95% alcohol had removed the active principle and left the powder impotent.

RESIDUE FROM ETHER-CHLOROFORM-AMMONIA EXTRACTION

Exper. 30.—Twenty-one days after the close of the preceding experiment, Rabbit 12 was given the dried residue from 50 gm. of extracted leaf powder, mixed with chopped vegetables, at the rate of 2 gm. per day. It ate the material readily and retained a good appetite and its normal condition without any manifestation of ill effects whatever.

In this case, the animal ate the residue from more than 3 lethal doses of the extract without harmful results, whereas the extracts when fed to Rabbits 13, 16 and 18 proved fatal (*Expers. 15, 16 and 17*).

Clearly, the extraction with ether-chloroform and ammonia had removed the active ingredient and left the residue inert.

RESIDUE FROM SALT SOLUTION EXTRACTION

Exper. 31.—An attempt was made in this instance to feed Rabbit 13 with the residue from the salt solution extraction, but with practical failure. The animal refused to eat anything which carried the residue, and after 8 days of persistent trial, during which period the rabbit lost 400 gm., the experiment was discontinued.

The fact that the extract from this material could not be proven to be poisonous (*Exper. 14*) and that the rabbit showed such a decided dislike for the powder, suggests that the latter still contained an active ingredient which had not been removed by the salt solution.

ORIGINAL UNTREATED LEAF POWDER

Exper. 32.—At the same time that we were conducting *Expers. 29 and 31*, we endeavored to feed Rabbit 11 with the original potent leaf powder which had been employed earlier in *Expers. 4, 5, 6, 7 and 8* with fatal results. The animal refused all food which contained the material and ate almost nothing for 8 days; accordingly the experiment was discontinued.

One cannot help being impressed with the fact that the rabbits seem to be able to distinguish between the residues from which the poisonous ingredient has been removed and those from which no demonstrable poison has

been secured. Whether they are able to discern this difference by intuition, taste or smell is difficult to say, but the fact remains unchanged, that they do eat the residues from which a demonstrable poisonous ingredient has been extracted and refuse all others.

FEEDING EXPERIMENTS WITH GUINEA-PIGS AND EUPATORIUM LEAF POWDER

Exper. 33.—It was shown in a number of our earlier experiments that the leaf powder from Morris, Ill., is poisonous for rabbits; its action upon guinea-pigs was studied by feeding Pig 3, 50 gm. in 2-gm. doses over a period of 25 days. It ate the powder readily when mixed with chopped carrots—quite the opposite from rabbits. Its weight decreased but little more than the control during the experimental period, and no apparent injury followed the ingestion of the snake-root. Weight at the beginning, 475 gm.; at the end, 391 gm.; loss, 84 gm.

Exper. 34.—This is a duplicate of the last experiment. In this case Pig 4 was given 50 gm. leaf powder in 2-gm. portions at 24-hour intervals. It remained perfectly well thruout the feeding, and at the conclusion of the experiment, no harmful effect of the powder could be noted.

Weight at the beginning, 489 gm.; at the end, 421 gm.; loss, 77 gm.

The last two experiments are of particular interest in that they confirm the results of *Exper. 26*, wherein a guinea-pig was fed the ether-chloroform-ammonia extract from 45 gm. of leaf powder (sufficient to kill 3 rabbits) without any apparent injury.

The fact seems well established from these results that the dried Eupatorium leaves contain nothing which is poisonous for guinea-pigs.

Exper. 35.—Pig 2 was carried as a control in connection with the two preceding experiments. It received the same kind and amount of food as Pigs 3 and 4 except without the addition of the leaf powder. All 3 animals were on a rather short ration as no more food was offered them than they would eat up completely, for we desired to have all of the leaf powder consumed. Under the conditions of the experiment, this control pig lost 62 gm.

SUMMARY

Both the fresh, green Eupatorium urticaefolium and the dried leaf powder contain an active ingredient which is poisonous for rabbits.

The active poisonous principle is present in plants grown in the greenhouse, as well as under natural out-of-door conditions.

The active constituent is soluble in 95% alcohol, and its solution yields a solid extract on evaporation which is poisonous for rabbits, but not for guinea-pigs.

The active ingredient is soluble in a mixture of ether-chloroform and ammonia, and its solution yields a solid extract on evaporation which is poisonous for rabbits, but not for guinea-pigs.

The active ingredient is not yielded by extraction with physiologic salt solution.

The active principle is present in the leaves, but not, or only sparingly so, in the stems and roots of dried plants.

There is no indication of anaphylaxis.

So far as is shown by these experiments, neither the leaf powder nor the different extracts are poisonous for guinea-pigs.

The viscera and meat from rabbits which had died from Eupatorium poisoning, when fed to a cat, were without harmful action.

No difference in poisonous properties could be noted between plants from a "milksick" and "nonmilksick" area.

The fatal dose of the leaf powder differs with the different animals, ranging from 4-17 gm.; whereas, the ether-chloroform-ammonia extract from 15 gm. given in 3 doses 24 hours apart invariably caused death in 4-6 days.

Rabbits suffering with *Eupatorium* poisoning usually manifest the first symptoms on the 3rd or 4th day (E. C. A. extr.). They refuse to eat, sit humped up, eyes half-closed and often keep the body swaying from side to side; the respiration is usually shallow, rapid and jerky; on the following day (4th or 5th) a flaccid paralysis of the head, neck and front legs ordinarily develops; this is followed by complete prostration and death in 24-36 hours.

The principal pathologic changes occur in the kidney, liver and heart where fatty degeneration and hyperemia are very marked.

Poured agar plates made from the heart blood, liver and kidneys were invariably sterile.

It is not intended to claim that all cases of disease with the symptoms of trembles or milksickness are due to the ingestion of the toxic substance present in *Eupatorium* leaves. Jordan and Harris have shown that a disease with similar if not identical symptoms occurs in a region in New Mexico where *Eupatorium* is not present.

OBSERVATIONS ON THE RATE OF GROWTH OF B. COLI

RAYMOND C. SALTER

*From the Bacteriological Laboratories of Engineering Experiment Station, Iowa State
College, Ames, Iowa*

This work was planned originally to determine quantitatively the effect of anilin dyes and of different mediums employed for the isolation of B. coli on the rate of growth of this organism.

The studies undertaken include:

1. The normal rate of growth of B. coli in peptone water at 37 C.
2. The effect of the age of the culture on the rate of growth.
3. The effect of crystal violet and brilliant green.
4. The rate of growth in various standard mediums.
5. The effect of varying amounts of bile salts.
6. The effect of varying amounts of peptone.
7. The effect of dipotassium phosphate.
8. The effect of Liebig's beef extract.

HISTORICAL

The work of several investigators has shown that the rate of growth of an organism varies during the life of the culture when kept under constant conditions of temperature, light, and other factors of environment. These variations in the rate of growth divide the life of the culture into distinct periods.

Buchanan¹ divides the life cycle of a culture into 7 phases, each phase having a different rate of growth per organism than the phase next preceding it.

The phases are as follows:

1. The initial stationary phase. During this phase the number of bacteria remains constant or nearly so.
2. The lag phase or positive growth acceleration phase. During this phase the average rate of growth per organism increases with the time.
3. The logarithmic growth phase. The rate of growth per organism in this period is constant. The organisms are dividing regularly and the number of organisms in the culture is increasing in a geometric ratio.
4. The phase of negative growth acceleration. The average rate of growth per organism decreases during this period.

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¹ Jour. Infect. Dis., 1918, 23, p. 109.

5. The maximum stationary phase. There is little or no increase or decrease in the number of organisms during this period.

6. The phase of accelerated death. The number of bacteria decreases slowly at first, but the rate of death per organism gradually increases until it reaches a maximum.

7. The logarithmic death phase. During this phase the rate of death per organism is constant.

From these observations, it is apparent that any investigation of the rate of growth of organisms must take into account these various growth phases. A given factor may influence the rate of growth in one phase and not in another. It may cause a lengthening of the lag phase and have no influence on the logarithmic growth phase, or may even stimulate growth during the latter phase.

Barber² has shown that the age of the culture influences the lag phase. He found that if a subculture is made in the same kind of medium during the logarithmic period, this subculture does not go through a lag period, but continues to grow at the same rate per organism as the parent culture. If the subculture is made after the logarithmic period, however, there is distinct lag. Penfold³ and later Chesney⁴ confirmed these observations. The lag phase, that is, the time from the inoculation to the beginning of the logarithmic phase, according to these investigators, includes the first two phases as outlined by Buchanan.

Rahn⁵ found that an increase in the numbers caused a shortening of the lag phases, when comparatively small numbers were used for inoculation.

Chick⁶ concludes from experiments with *B. coli* that the previous history of the culture greatly influences the rate of growth during the initial period. She found that harmful effects were produced by prolonged incubation of the culture at 37 C., or by exposure to borax, or to fresh milk. Injury to the cells in various ways caused a period of lag when the organisms were transferred to a favorable medium.

Lane-Clayton⁷ determined the generation time for *B. coli* and for *B. enteritidis* (Gaertner) at various temperatures. He found that generation time is shortened by increases in temperature up to 42 C., and that these variations in rate of growth follow the Arrhenius-Van't Hoff law. His results in regard to generation time of *B. coli* were in accord with those of Barber.²

METHOD

The method of measuring the effect of various factors on the rate of growth of organisms was suggested by the frequently reported observation of investigators that the rate of growth per organism during the logarithmic phase is constant. If the logarithms of the numbers of viable organisms during intervals of this period are plotted against time, the logarithms fall along an ascending straight line. A given strain of organism should give similar curves if the conditions of growth are exactly reproduced in each case. With this

² Jour. Infect. Dis., 1908, 5, p. 379.

³ Jour. Hyg., 1914, 14, p. 215.

⁴ Jour. Exper. Med., 1916, 24, p. 387.

⁵ Centralbl. f. Bakteriol., 1900, 16, p. 417.

⁶ Jour. Hyg., 1912, 12, p. 414.

⁷ Jour. Hyg., 1909, 9, p. 239.

fact well established, the effect of various factors might be noted by the change which they produce in the slope of the line. We would expect a stimulating substance to increase the slope and an inhibiting substance to decrease the slope of the line. In other words, the tangent of the angle which the curve makes with the X axis, compared with the normal, could be taken as a measure of the influence of the substance or factor.

Perhaps a more convenient method of measuring and expressing the effect of the factors on the rate of growth is that used by Chesney.⁴ He expressed the rate of growth in terms of average generation time. The generation time is the time required by an organism to grow to maturity and divide. In other words, it is the time required to produce a single generation. This can be computed by a formula worked out by Buchner, Longard, and Riedlin.⁸

It is assumed that during the logarithmic period the organisms are increasing in a geometrical ratio. By the law of geometrical proportions: $b = B 2^n$, where B is the number of bacteria at the beginning and b is the number of bacteria after time t, and n is the number of generations. If there have been

n generations in time t, then $g = \frac{t}{n}$, where g is generation time.

$$\text{Then } n = \frac{t}{g}$$

$$\text{and } b = B 2^{\frac{t}{g}}$$

Applying logarithms to this equation:

$$\text{Log } b = \text{log } B + \text{log } 2^{\frac{t}{g}}$$

$$g = \frac{t \log 2}{\log b - \log B}$$

When time t is not too long the generation time can be estimated with considerable accuracy, but since the rate of growth per organism changes at the beginning and at the end of the logarithmic phase, the number of organisms must be determined at frequent intervals.

The method employed was to grow the organisms at a constant temperature in a simple liquid medium, determine the numbers present at frequent intervals of time, and compute the generation time. The effect of variations in the medium and other factors could then be determined by the change which they produce in the generation time. The advantage of this method is that it shows on which phase of the life of the culture the effect is most marked.

The medium chosen for this work was a one-half per cent. Difco peptone-distilled water solution. All of the peptone for these experiments was taken from the same bottle to insure uniformity in composition. It was sterilized in test tubes in the autoclave at 15 pounds pressure for 15 minutes. This medium was stored in an icebox, but never kept more than 3 or 4 days on account of change in concentration due to evaporation.

The organism used was a well tested strain of *B. (coli) communis*. This was grown in the peptone water and transferred daily for 3 successive days before each experiment. This was done to avoid any changes in the rate

⁴ Centralbl. f. Bakteriöl., 1887, 11, p. 1.

of growth due to previous environment of the culture. Each experiment was begun with a 22-24 hour culture, except where otherwise stated.

The temperature of incubation during these experiments was kept constant by means of a DeKhotinsky electrically controlled water-bath. By the use of this apparatus the temperature could easily be kept constant within 1 degree.

The determinations of the number of organisms present at various periods of growth were made by plating on plain agar. This medium was prepared in large quantities and great care taken to insure uniformity of composition. Four plates were made for each determination. Dilutions, when necessary, were made in sterile tap water. The exposure to the water was never for more than a few minutes and apparently it had no effect on the subsequent development of the organism.

The plates were counted after 48 hours of incubation at 37° C. In making the counts a hand lens was used which magnified about 5 diameters. All plates which could not be counted accurately were discarded.

THE RATE OF GROWTH OF *B. COLI* IN PEPTONE WATER

An experiment was planned to determine the rate of growth per organism of *B. coli* in peptone water and to determine roughly the limits of the logarithmic period as a working basis for following experiments. Four tubes, each containing 10 cc of peptone water, were inoculated: 2 with *B. communis* and 2 with *B. communior*. One-tenth cc of a 1:10,000 dilution of a 24-hour culture was added in each tube. This gave between 200 and 300 organisms per cc. These were incubated in the water-bath and counts made at the beginning and at the end of each 3-hour period. The results are shown in Table 1.

TABLE 1
RATE OF GROWTH OF *B. COLI* IN PEPTONE WATER

	Time in Minutes	Average Number Bac- teria per C C	Generation Time in Minutes
<i>B. communis</i>	0	175	
	180	4,550	38.5
	360	639,000	25.3
	540	57,100,000	27.8
<i>B. communis</i>	0	158	
	180	5,600	35.0
	360	585,000	26.8
	540	48,000,000	28.4
<i>B. communior</i> *	0	83	
	180	1,350	44.8
	360	379,000	22.1
	540	44,500,000	26.2

* The plates of the second culture of *B. communior* showed contamination and, therefore, the results are not included in the table.

It will be noted that in each case the first 3 hours show considerably slower growth than the following periods, due to a lag phase. The fact that the last 3 hours always show a lengthening of the generation time would indicate that this time included a part, at least, of the phase of negative growth acceleration.

The mean generation time for *B. coli* at 37 C., as determined by Barber² was 17-21 minutes. Lane-Clayton⁷ reports similar results. Both these investigators used plain broth as a culture medium.

In order to get a better view of the various phases of growth under the conditions of this experiment, it was repeated using *B. communis* and making a count every 2 hours. This experiment was continued for 12 hours. The results are shown in Table 2.

TABLE 2
RATE OF GROWTH OF *B. COLI* IN PEPTONE WATER

Time in Minutes	Average Number Bacteria per C C	Generation Time in Minutes
0	233	
120	600	88.0
240	79,450	17.2
360	1,360,000	29.3
480	22,200,000	29.8
600	448,000,000	27.8
720	1,489,000,000	69.4

The 2-hour period shows the slow rate of growth at the beginning more distinctly since it more nearly represents the initial and lag phases. The very short generation time between the 2nd and 4th hours is probably an experimental error. Repeated counts in subsequent experiments failed to show such a short period of rapid growth as this figure would indicate. The rate of growth in peptone water at 37 C. is apparently constant from the 4th to the 10th hour after inoculation. After the 10th hour the lengthening of the generation time is quite marked.

THE EFFECT OF AGE OF CULTURE

As previously stated, Barber² noted that subcultures from cultures which are still in the logarithmic phase, show no initial stationary or lag phase. An experiment was made to check this observation. Subcultures were made from rapidly growing cultures after 4 hours of incubation and cultures and subcultures were counted at the end of 4 additional hours. The results shown in Table 3 would indicate the rate of growth of the subcultures closely approximates that of the parent culture.

It is apparent that the age of the culture influences the rate of growth within certain limits. In order to determine the best age of

culture for the experiments which follow, the generation times were determined for 6 cultures varying in age from 1-6 days. The cultures had been kept in the incubator at 37 C. The results of this experiment (Table 4) are quite striking. The variations in the generation time with cultures of varying ages appear to be almost entirely within the first 2 hours. A glance at the generation times in this period will show that the stationary and lag phases are not greatly increased until the 3rd day is passed and then the increase in these phases is very decided.

TABLE 3
THE RATE OF GROWTH OF SUBCULTURES MADE DURING THE LOGARITHMIC PHASE

Time in Hours	Culture	Average Number Bacteria per C C	Generation Time in Minutes
0	<i>B. communis</i>	263	
4		10,900	42.5
8		2,980,000	31.5
4	Subculture	1,000	
8		144,000	36.1
0	<i>B. communis</i>	305	
4		9,450	51.5
8		2,570,000	31.5
4	Subculture	945	
8		238,500	31.9
0	<i>B. communior</i>	230	
4		56,200	32.2
8		18,195,000	30.6
4	Subculture	5,620	
8		2,625,000	28.8
0	<i>B. communior</i>	231	
4		19,500	39.8
8		5,022,000	33.2
4	Subculture	1,950	
8		890,000	28.9

If we accept the view of Coplans⁹ that the slow rate of initial growth is due to previous injury to the cells caused by products of metabolism, it would seem that some decided change has taken place in the culture between the 3rd and 4th days.

Buchanan's explanation of the lag is that bacterial cells, like seeds of higher plants, go into a resting stage and when placed again in favorable environment, they require a certain length of time in which to germinate. Interpreting the results from this viewpoint, we must

⁹ Jour. Pathol., 1909, 14, p. 1.

TABLE 4
THE EFFECT OF AGE OF CULTURE ON RATE OF GROWTH OF *B. COMMUNIS*

Time in Hours	Age of Culture	Average Number Bacteria per C C	Generation Time in Minutes
0	One Day	388	
2		1,070	72.1
4		13,000	33.3
6		177,000	31.8
8		4,400,000	25.9
0	Two Days	538	
2		1,520	80.1
4		19,500	32.6
6		550,000	24.9
8		9,800,000	28.9
0	Three Days	338	
2		930	82.2
4		29,700	24.0
6		680,000	26.6
8		6,100,000	37.9
0	Four Days	349	
2		520	179.7
4		7,500	26.9
6		167,000	23.1
8		1,750,000	30.5
0	Five Days	271	
2		461	134.9
4		5,300	29.4
6		77,500	26.7
8		800,000	30.7
0	Six Days	262	
2		330	310.6
4		6,800	23.7
6		93,000	27.5
8		1,420,000	26.2

SUMMARY OF GENERATION TIMES

Time in Hours	One Day	Two Days	Three Days	Four Days	Five Days	Six Days
0-2	72.1	80.1	82.2	179.7	134.9	310.6
2-4	33.3	32.6	24.0	26.9	29.4	23.7
4-6	31.8	24.9	26.6	23.1	26.7	27.5
6-8	25.9	28.9	37.9	30.5	30.7	26.2

conclude that a large number of cells go into the resting stage after the 3rd day. This explanation is quite as reasonable as that based on Coplans' theory.

In order to get at the changes which take place in cultures during the initial phases, the generation times of 1- and of 6-day cultures were determined at 30 minutes' intervals, with the results shown in Table 5. The 6-day culture shows both a lengthening of the lag period and an increase in the generation time during this period over the 1-day culture. The 1-day culture has nearly reached its maximum

TABLE 5
THE EFFECT OF AGE OF CULTURE ON RATE OF GROWTH OF *B. COMMUNIS*

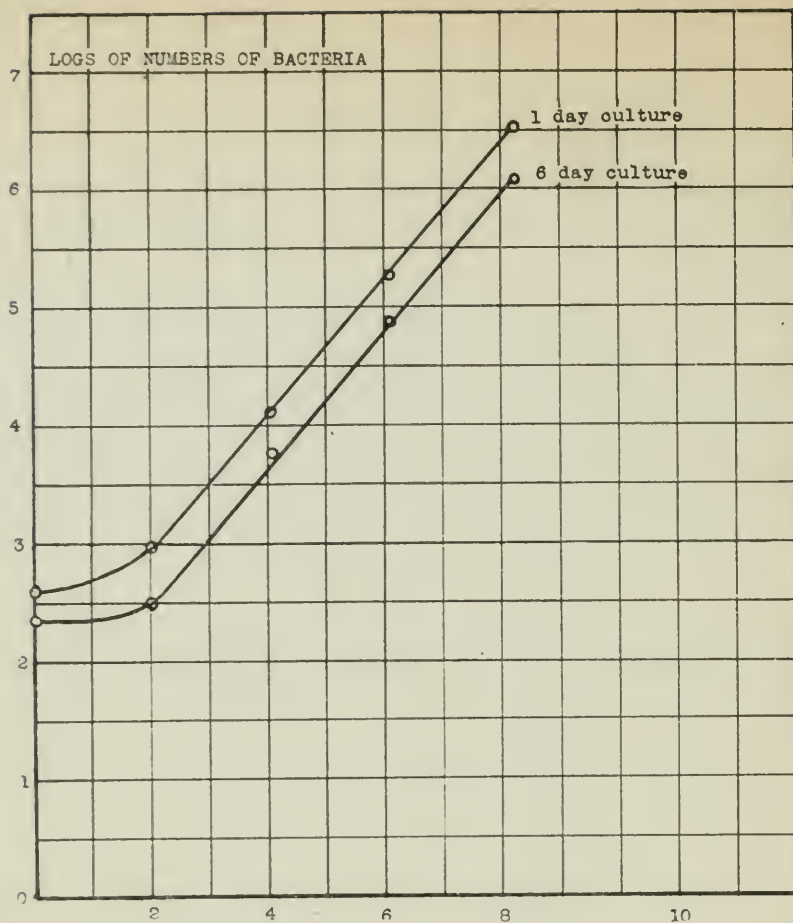
Time in Minutes	Age of Culture	Average Number Bacteria per C C	Generation Time in Minutes
0	One Day	30	
30		37	99.1
60		55	52.4
90		105	32.2
0	Six Days	37	
30		41	202.4
60		52	87.5
90		70	69.9

SUMMARY OF GENERATION TIMES

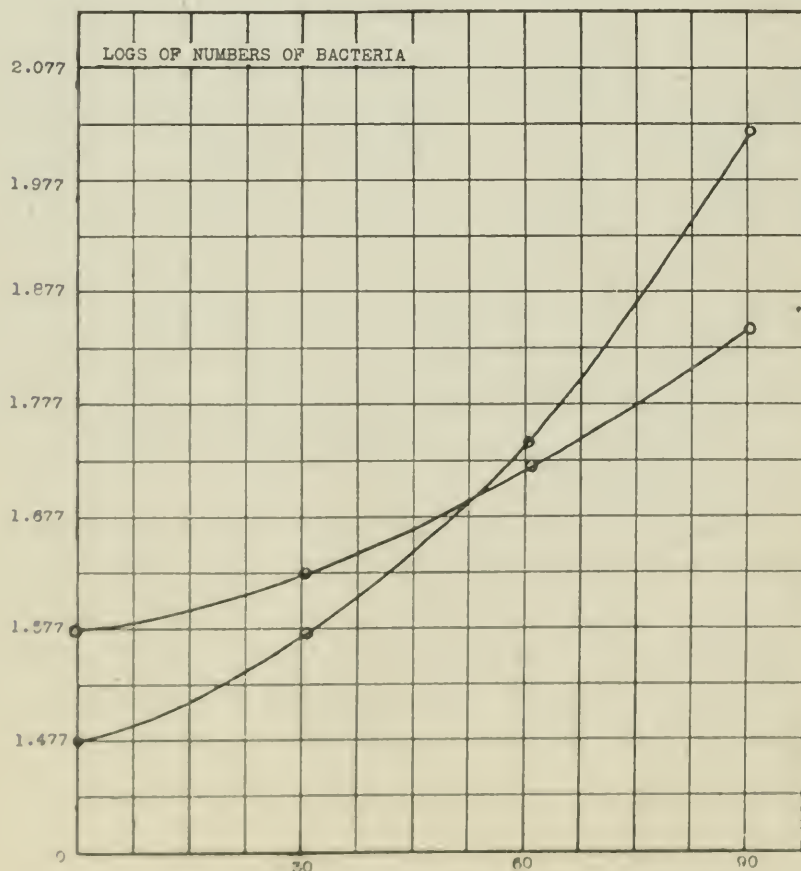
Time in Minutes	One Day	Six Days
0-30	99.1	202.4
30-60	52.4	87.5
60-90	32.2	69.9

rate of growth in 1 hour. Graphs 1 and 2 illustrate the difference in the rates of growth of the 2 organisms. Graph 1 shows the constancy of the rate of growth of cultures after the lag period and shows also that the rate of growth during the logarithmic period is independent of the age of the culture.

Graph 2 shows that in the 1-day culture there is growth from the beginning, and if there is a stationary phase at all, it must be extremely short. The 6-day culture also shows growth from the beginning, but the rate of acceleration of growth is decidedly slower than in the 1-day culture. From the results of this experiment it seemed that a 24-hour culture would be best to use for succeeding experiments.



Graph 1.—Showing the rate of growth of a one- and six-day culture.



Graph 2.—Effect of age of culture on lag.

THE EFFECT OF CRYSTAL VIOLET AND BRILLIANT GREEN

It has been known for some time that anilin dyes effect the growth of bacteria. Billroth was the first to note this effect, although Stilling¹⁰ was the first to use anilin dyes in a practical way as antiseptics. Churchman¹¹ and others have noted that certain dyes have a selective action on bacteria.

Gentian violet, according to Churchman,¹¹ can be used to divide bacteria roughly into 2 groups, one of which will grow readily in the presence of this dye, and the other will be entirely inhibited by it. The first group corresponds to the gram-negative bacteria and the second to the gram-positive bacteria. Charles Krumweide and Josephine Pratt¹² confirmed this work of Churchman's on the selective action of dyes. They found that occasionally a gram-negative organism refused to grow on violet agar, often differentiating it from members of the same species or variety. They found a marked difference in the growth of dysentery and paradysentery, especially on fuchsin agar. Diphtheria and diphtheroid bacteria acted differently in the presence of naphthalamine blue.

All of this work was based on the growth of the organism on solid mediums containing the dye, and the effect of the dye was read by the relative abundance of growth on the dye agar compared with the plate agar controls.

Kriegler¹³ attempted to find some correlation between the chemical structure of the dyes and their disinfecting properties. He also noted a marked variation in the resistance of closely related organisms.

Nowhere in the literature was found any attempt at an accurate quantitative measure of the effect of dyes on growth. If great variations in the lethal dilutions for related organisms exist, and if distinctions can be readily made between growth of related organisms on agar cultures containing the dyes, it would seem that an accurate quantitative measure of the inhibiting effect of weak dilutions of dyes might be of value.

CRYSTAL VIOLET

A stock 10% solution of the dye in distilled water was prepared and from this a series of dilutions in peptone water was made. The stock solution was tightly stoppered and kept in the ice-box to prevent evaporation. The dilutions in peptone water were prepared fresh for each experiment.

¹⁰ Berlin. klin. Wchnschr., 1890, 24; Arch. f. Exper. Path. u. Pharmacol., 1891, 28, p. 357.

¹¹ Jour. Exper. Med., 1913, 17, p. 373.

¹² Jour. Exper. Med., 1914, 19, p. 20 and p. 501.

¹³ Centralbl. f. Bakteriol., I, O., 1911, 59, p. 481.

Tubes containing 10 cc of the various dilutions of dyes in peptone water, and one control tube containing peptone water only, were placed in the water bath for 15 minutes previous to making inoculations in order to bring the mediums to proper temperature. At the end of 4 hours' growth, a count was made on all of the tubes and a second series of the same dilutions of dyes was inoculated from the control tube. This was to determine whether the effect of the dye would be the same on a rapidly growing culture as on the fresh transfers. At the end of 8 hours, the number of bacteria per cc in all tubes was determined. Several preliminary experiments failed to give good results until the proper dilution of dyes and the proper dilutions for the plate counts were established. It was found that growth took place in dilutions of 1:200,000. In strong dilutions growth was very irregular or absent.

It was noted that growth appeared in a particular dilution on one day while on another day, it was absent or very slight. It would appear that the action of the dyes varied on different days when all controllable environmental factors seemed to be constant. The action of the dyes seemed to be strongest on bright sunshiny days. It was suggested that the variable factor might be the light.

Table 6 shows a comparison of generation times on different days. The fact that the rate of growth of the controls is constant on different days within the limit of experimental error, would point to the fact that the dyes themselves were more active on one day than on another. It was suggested that the action of varying amounts of light on a fluorescent dye might influence its growth inhibiting power.

TABLE 6
GENERATION TIME ON DIFFERENT DAYS

Dilution of Dye	Period of Growth in Hours	Nov. 6	Nov. 8	Nov. 14	Nov. 22	Nov. 28	Dec. 8	Dec. 13
Control, no dye	0-4	35.2	35.7	35.4	37.0	34.8	32.8
	4-8	30.1	27.3	26.8	25.1	27.3	28.9
1:75,000	0-4	decrease	569.0					
	4-8	decrease	decrease					
1:100,000	0-4	decrease	477.0					
	4-8	30.2	131.3					
1:200,000	0-4	90.6	96.8	32.6	62.0	124.1
	4-8	47.4	92.9	41.6	44.2
1:400,000	0-4	58.7	55.8	53.3	49.6	44.2	55.6
	4-8	27.8	77.1	36.9	61.5	55.0	33.0
1:600,000	0-4	40.5	51.1	59.3	39.8	38.4
	4-8	31.4	35.1	29.4	29.6	33.4
1:800,000	0-4	34.2	36.9	555.3	39.8	38.4
	4-8	28.8	41.1	37.5	29.6	333.3
1:1,000,000	0-4	56.0	37.4	36.5
	4-8	28.0	28.2	32.3

Von Tappeiner,¹⁴ Raab,¹⁵ and others have shown that organisms in water solutions of fluorescent dyes behave differently in the presence of light than they do in the dark. Raab investigated the effect of a

¹⁴ Deutsch. Arch. f. klin. Med., 1904, 80, p. 1904.

¹⁵ Inaug. Diss. München, 1900.

TABLE 7
THE EFFECT OF CRYSTAL VIOLET ON THE RATE OF GROWTH OF B. COMMUNIS

Time in Hours	Dilutions of Dyes	Average Number of Bacteria per C C	Generation Time in Minutes
0	All	393	
4	Control	46,300	34.8
8	Control	17,400,000	27.2
4	1:200,000	5,740	62.0
8	1:200,000	304,000	41.6
4	1:400,000	17,000	44.2
8	1:400,000	348,000	55.0
4	1:600,000	208,000	41.7
8	1:600,000	4,880,000	30.5
4	1:800,000	25,600	39.8
8	1:800,000	7,080,000	29.6
4	1:1,000,000	31,400	37.4
8	1:1,000,000	11,400,000	28.2
4	Subculture 1:200,000	34,800	67.0
4	Subculture 1:400,000	82,800	57.6
4	Subculture 1:600,000	81,000	58.0
4	Subculture 1:800,000	86,000	52.7
4	Subculture 1:1,000,000	127,000	50.2

SUMMARY OF GENERATION TIMES

Time	1:200,000	1:400,000	1:600,000	1:800,000	1:1,000,000	Control
0-4	62.0	44.2	41.7	39.8	37.4	34.8
4-8	41.6	55.0	30.5	29.6	28.2	27.2
Subculture	67.0	57.6	58.0	52.7	50.0	

TABLE 8
THE EFFECT OF CRYSTAL VIOLET ON THE RATE OF GROWTH OF B. COMMUNIS

Time in Hours	Dilutions of Dyes	Average Number of Bacteria per C C	Generation Time in Minutes
0	All	390	
4	Control	62,500	32.8
8	Control	19,750,000	28.9
4	1:200,000	1,490	124.1
8	1:200,000	64,300	44.2
4	1:400,000	10,410	50.6
8	1:400,000	1,710,000	33.0
4	1:600,000	20,300	42.7
8	1:600,000	3,120,000	33.3
4	1:800,000	29,800	38.4
8	1:800,000	4,400,000	33.3
4	1:1,000,000	37,000	36.5
8	1:1,000,000	6,350,000	32.3
4	Subculture 1:200,000	34,900	96.7
4	Subculture 1:400,000	111,000	57.8
4	Subculture 1:600,000	174,000	50.0
4	Subculture 1:800,000	200,000	48.0
4	Subculture 1:1,000,000	274,000	44.0

SUMMARY OF GENERATION TIMES

Time	1:200,000	1:400,000	1:600,000	1:800,000	1:1,000,000	Control
0-4	124.1	50.6	42.7	38.4	36.5	32.8
4-8	44.2	33.0	33.3	33.3	32.3	28.9
Subculture	96.7	57.8	50.0	48.0	44.0	

number of fluorescent substances on various organisms. He found that many organisms were killed when exposed to daylight in fluorescent solutions, while in the dark they lived indefinitely. Von Tappeiner found that fluorescent dyes will also destroy the activity of toxins and antitoxins when exposed to the light. After a somewhat extended study of fluorescent substances, Von Tappeiner concluded that the photodynamic effect is associated with those rays which are absorbed by the fluorescent materials. He also concluded that in order to bring about the photodynamic action, the material in question must be visibly fluorescent even when examined in the rays of a mercury vapor lamp.

It would seem then that an explanation of the variations in the rate of growth on different days based on the photodynamic action of the dyes when exposed to lights of different intensity, would be inadequate without further experimental evidence.

Tables 6 and 7 show the results of two experiments with crystal violet which are quite comparable. It will be noted that the dye has its greatest inhibiting power during the first 4 hours. After that the figures for the generation times are more nearly like those of the control. Evidence that the dyes are active in producing a lag phase is shown by the fact that the subcultures from the controls into the dyes do not grow at nearly the same rate as the cultures which have been exposed to the dyes for a 4-hour period. In most cases the lag produced in the subcultures is greater than the lag produced in the 24-hour cultures from which the first inoculations were made. It would seem that the actively growing cells were more susceptible to injury than the cells in an older culture.

BRILLIANT GREEN

Two experiments were made with brilliant green, identical with those performed with crystal violet, except that higher dilutions of the dye were used. Preliminary experiments showed this dye to be much more inhibitive than crystal violet. The results are shown in Tables 9 and 10. In general, they are the same as those for stronger solutions of crystal violet. Brilliant green does not seem to affect the lag period more than it does the logarithmic period, but it does produce a marked lag in subcultures from an actively growing culture, as did the crystal violet.

TABLE 9

THE EFFECT OF BRILLIANT GREEN ON RATE OF GROWTH OF B. COMMUNIS

Time in Hours	Dilutions of Dyes	Average Number of Bacteria per C C	Generation Time in Minutes
0	All	274	
4	Control	72,000	28.0
8	Control	27,340,000	22.0
4	1:600,000	70	
8	1:600,000	55	
4	1:800,000	240	
8	1:800,000	245	826.0
4	1:1,000,000	550	299.0
8	1:1,000,000	1,750	144.0
4	1:2,000,000	2,940	129.0
8	1:2,000,000	1,700	
4	Subculture 1:600,000	1,160	
4	Subculture 1:800,000	26,700	91.0
4	Subculture 1:1,000,000	12,000	325.0
4	Subculture 1:2,000,000	178,000	55.8

SUMMARY OF GENERATION TIMES

Time	1:600,000	1:800,000	1:1,000,000	1:2,000,000	Control
0-4	No growth	No growth	239	129	28
4-8	No growth	826	144	...	22
Subculture	No growth	91	325	55.8	

TABLE 10

THE EFFECT OF BRILLIANT GREEN ON RATE OF GROWTH OF B. COMMUNIS

Time in Hours	Dilutions of Dyes	Average Number of Bacteria per C C	Generation Time in Minutes
0	All	213	
4	Control	70,000	28.7
8	Control	29,000,000	27.6
4	1:2,000,000	5,630	50.8
8	1:2,000,000	24,000	113.1
4	1:4,000,000	6,600	45.4
8	1:4,000,000	81,000	66.3
4	1:6,000,000	14,400	39.4
8	1:6,000,000	1,390,000	36.3
4	1:10,000,000	61,600	29.3
8	1:10,000,000	34,900,000	26.2
4	Subculture 1:2,000,000	37,900	99.9
4	Subculture 1:4,000,000	47,000	87.3
4	Subculture 1:6,000,000	121,000	58.3
4	Subculture 1:10,000,000	322,000	43.4

SUMMARY OF GENERATION TIMES

Time in Hours	1:2,000,000	1:4,000,000	1:6,000,000	1:10,000,000	Control
0-4	50.8	45.4	39.4	29.3	28.7
4-8	113.1	66.3	36.3	26.2	27.6
Subculture	99.9	87.3	58.3	43.4	

Both crystal violet and brilliant green, because of their inhibiting influence on the members of the colon group and other organisms, are used in the isolation of members of the typhoid group. Further investigation of these dyes concerning their effect on other members of the colon group should be made. The present work will be confined to *B. (coli) communis* and a study of the other factors on its rate of growth.

TABLE 11
THE EFFECT OF VARIOUS CULTURE MEDIUMS ON RATE OF GROWTH OF *B. COMMUNIS*

Time in Hours	Medium	Average Number Bacteria per C C	Generation Time in Minutes
0	$\frac{1}{2}\%$ Peptone	282	
2		1,490	49.9
4		44,100	24.5
8		20,800,000	27.0
2	Peptone K_2HPO_4	4,200	30.8
4		192,000	21.7
8		38,100,000	31.4
2	Peptone bile salt
4		10,600	45.9*
8		1,510,000	33.4
2	Standard broth	2,210	43.9
4		76,000	23.5
8		40,500,000	41.8
2	Lactose broth	1,300	54.6
4		37,800	22.1
8		33,700,000	26.0

SUMMARY OF GENERATION TIMES

Time in Hours	$\frac{1}{2}\%$ Peptone	Peptone K_2HPO_4	Peptone Bile Salt	Standard Broth	Lactose Broth
0-2	49.9	30.8	43.9	54.6
2-4	34.5	21.7	45.9*	23.5	22.1
4-8	27.0	31.4	33.4	41.8	26.0

* Determined for 4 hour period.

THE EFFECT OF VARIOUS STANDARD MEDIUMS

A large number of liquid mediums are used for the cultivation of *B. coli*, for preliminary enrichment, isolation, presumptive tests, and tests for the presence of metabolic products. The rates of growth in a few of these mediums were determined in order to detect the presence of inhibiting or stimulating substances and to get a comparison of the rates of growth.

The following mediums were used:

1. Peptone water: One-half per cent. peptone in distilled water.
2. Peptone-dipotassium phosphate: One-half per cent. peptone water plus two-tenths per cent. dipotassium phosphate.
3. Peptone bile salt: One-half per cent. peptone to 1% of commercial bile salt.
4. Broth: Prepared according to "Standard Methods of Water Analysis," except that the reaction was made neutral to phenolphthalein before sterilization.
5. Lactose broth: Prepared according to "Standard Methods of Water Analysis."

TABLE 12
THE EFFECT OF VARIOUS MEDIUMS ON RATE OF GROWTH OF B. COMMUNIS

Time in Hours	Medium	Average Number Bacteria per C C	Generation Time in Minutes
0	Standard peptone	225	
2		875	61.5
4		27,200	24.3
8		22,800,000	25.5
2	Peptone K ₂ HPO ₄	1,790	40.3
4		95,500	21.0
8		52,200,000	27.2
2	Peptone bile salt	700	73.8
4		13,800	28.0
8		9,250,000	26.4
2	Standard broth	960	57.4
4		46,900	21.4
8		37,100,000	25.7
2	Lactose broth	875	61.5
4		38,500	22.1
8		26,400,000	26.3

SUMMARY OF GENERATION TIMES

Time in Hours	½% Peptone	Peptone K ₂ HPO ₄	Peptone Bile Salt	Standard Broth	Lactose Broth
0-2	61.5	40.3	73.8	57.4	61.5
2-4	24.3	21.0	28.0	21.4	22.1
4-8	25.5	27.2	26.4	25.7	26.3

The hydrogen-ion concentrations of all these mediums were determined by colorimetric comparison with standards, and they were all found to have a P_H value of between 6.5 and 7.6. This amount of variation is probably within the favorable range of concentration for the growth of B. coli, or at least these concentrations would not be likely to cause any inhibitions of growth. Clark has some unpublished work on the effect of hydrogen-ion concentration. In a personal letter to Prof. Max Levine, Jan. 24, 1918, he states that "the most striking results which we obtained were, first: the broad P_H zone within which the maximum rate of reproduction during the period of logarithmic increases was practically constant."

Tables 11 and 12 contain the results of duplicate experiments to show the rate of growth of *B. coli* in various mediums. A glance at the maximum numbers of organisms reached in the various mediums will show that the standard broth and the peptone phosphate mediums yield the most abundant growth, while the lactose broth, peptone water, and the bile salt mediums follow in the order named. It is apparent that beef extract and dipotassium phosphate greatly stimulate the growth of *B. communis*. In the case of the dipotassium phosphate this stimulation appears to be quite marked in the lag period, while in the standard broth and lactose broth, whose stimulating ingredient is undoubtedly the beef extract, the greatest stimulation appears during the second 2 hours of growth. This would indicate that the stimulating effects of dipotassium phosphate and beef extract were not identical, and that the stimulation of beef extract was not entirely due to its phosphate content.

A very decided inhibition of growth is caused by the addition of bile salt. Not only does it produce a lengthened lag period, but it increases the generation time throughout the period of growth. Since bile salts are used in the presumptive test for *B. coli*, a further study of the effect of this substance was made.

THE EFFECT OF BILE SALTS

Prescott and Winslow¹⁶ state: "By far the most satisfactory results in making a rapid test for the colon group have been obtained by the use of mediums containing bile salts." Dunschmann¹⁷ studied a favorable action of bile on *B. typhosus* and a slight inhibition on *B. coli*. Echer¹⁸ states: "Bile salts distinctly retard the growth of typhoid bacilli." Most of the work seems to have been done with whole bile rather than with bile salts. Hirokawa¹⁹ reports that *B. coli*, *B. typhosus*, and *B. Paratyphosus* A and B develop well in bile mediums. Frankel and Krause²⁰ report that human bile also stimulates the growth of typhoid bacilli, *B. coli*, *Staphylococcus pyogenes*, and *Vibrio cholerae*. On the other hand, Pies²¹ found a sample of human bile which inhibited both *B. coli* and *B. typhosus*. Nichols²² states that rabbit bile in vitro may be antiseptic to *B. typhosus*, *B. paratyphoid* A, *B. coli*, and *B. dysenteriae*. Jordan²³ asserts: "Bile inhibits at least from one-third to one-half of the viable cells of *B. coli* and sometimes a much larger proportion." Obst²⁴ similarly states that lactose bouillon permits the development of about twice as many coli as will lactose peptone ox-bile medium.

¹⁶ Principles of Water Bacteriology, 1913.

¹⁷ Ann. de l'Inst. Pasteur, 1909, 23, p. 29.

¹⁸ Jour. Exper. Med., 1918, 22, p. 95.

¹⁹ Centralbl. f. Bakteriol., I. O., 1909, 53, p. 12.

²⁰ Ztschr. f. Hyg., 1899, 32, p. 91.

²¹ Inaug. Diss. Strassburg, 1907.

²² Jour. Exper. Med., 1916, 24, p. 497.

²³ Jour. Infect. Dis., 1913, 12, p. 326.

²⁴ Jour. Bacteriol., 1916, 1, p. 73.

From the literature, it is apparent that there is lack of agreement regarding the effect of bile on B. coli and related organisms.

The present study was confined to different quantities of bile salts. Dilutions of commercial bile salt in peptone water were prepared and the generation times for B. coli for the various dilutions were determined. It is evident from the results of these experiments that small quantities of bile salts, that is, under five-tenths per cent., stimulate the growth of B. communis, while greater amounts, such as

TABLE 13
THE EFFECT OF DIFFERENT CONCENTRATIONS OF BILE SALT ON RATE OF GROWTH OF
B. COMMUNIS

Time in Hours	Per Cent. Bile Salt	Average Number Bacteria per C C	Generation Time in Minutes
0	All	124	
2	Control	410	70.0
4	Control	12,800	24.3
8	Control	14,600,000	23.7
2	0.1	595	58.7
4	0.1	17,900	24.6
8	0.1	23,700,000	23.2
2	0.2	515	58.7
4	0.2	17,900	23.5
8	0.2	36,600,000	21.9
2	0.3	450	64.8
4	0.3	17,000	23.0
8	0.3	24,700,000	22.8
2	0.5	520	58.2
4	0.5	20,200	22.8
8	0.5	21,500,000	23.9
2	0.7	425	67.8
4	0.7	13,400	24.2
8	0.7	10,800,000	24.9
2	1.0	435	66.7
4	1.0	7,150	29.9
8	1.0	1,650,000	30.5

SUMMARY OF GENERATION TIMES

Time in Hours	0.0	0.1	0.2	0.3	0.5	0.7	1.0
0-2	70.0	53.2	58.7	64.8	58.2	67.8	66.7
2-4	24.3	24.6	23.5	23.0	22.8	24.2	29.9
4-8	23.7	23.2	21.9	22.8	23.9	24.9	30.5

1%, greatly inhibit growth. This may, in a measure, explain the disagreement in the results of other investigators. The bile and bile salts from various sources undoubtedly contained varying amounts of the stimulating substances with the result that some found stimulation and others found inhibition. Several investigators have reported a selective action on the part of bile and bile salts, and there seems to be need of investigating the effect of varying amounts of bile salt on other members of the colon group.

THE EFFECT OF DIPOTASSIUM PHOSPHATE

As was noted in the foregoing, the addition of two-tenths per cent. of dipotassium phosphate to peptone water greatly stimulated growth. Clark and Lubs²⁵ recommend the use of a peptone dipotassium phosphate medium for testing for various reactions of *B. coli*. Levine²⁶ used dipotassium phosphate instead of beef extract in a modified Endo medium which he found very successful. The dipotassium phosphate acts as a hydrogen-ion buffer and obviates the necessity of careful adjustment of the reaction.

TABLE 14

THE EFFECT OF DIFFERENT CONCENTRATIONS OF BILE SALT ON THE GROWTH OF *B. COMMUNIS*

Time in Hours	Per Cent. Bile Salt	Average Number Bacteria per C C	Generation Time in Minutes
0	All	250	
2	Control	1,250	49.3
4	Control	31,200	25.9
8	Control	32,600,000	24.0
2	0.1	1,330	47.7
4	0.1	46,800	23.4
8	0.1	33,800,000	25.4
2	0.2	1,480	44.3
4	0.2	50,600	23.6
8	0.2
2	0.3	1,600	43.1
4	0.3	56,300	23.6
8	0.3	36,000,000	25.8
2	0.5	1,600	43.1
4	0.5	58,300	23.2
8	0.5	15,100,000	30.1
2	0.7	1,130	52.5
4	0.7	38,600	23.6
8	0.7	6,760,000	32.2
2	1.0	1,180	51.0
4	1.0	23,000	28.1
8	1.0	1,780,000	38.4

SUMMARY OF GENERATION TIMES

Time in Hours	0.0	0.1	0.2	0.3	0.5	0.7	1.0
0-2	49.3	47.7	44.3	43.1	43.1	52.5	51.0
2-4	25.9	23.4	23.6	23.6	23.2	23.6	28.1
4-8	24.0	25.4	25.8	30.1	32.2	38.4

An experiment was planned to determine what amount of dipotasium phosphate would give the most rapid rate of growth of *B. communis*. Various amounts of phosphate were carefully weighed and dissolved in portions of the same batch of peptone water. These solutions were then tubed and sterilized. The generation times were determined in the usual manner.

²⁵ Jour. Bacteriol., 1917, 2, p. 1.
²⁶ Jour. Am. Pub. Health Assn., 1918, 8, p. 866.

The results shown in Tables 15 and 16 would indicate that the rate of growth of *B. communis* is stimulated by dipotassium phosphate in amounts up to 1% in one-half per cent. peptone water.

TABLE 15
THE EFFECT OF DIPOTASSIUM PHOSPHATE (K_2HPO_4) ON RATE OF GROWTH OF *B. COMMUNIS*

Time in Hours	Per Cent. K_2HPO_4	Average Number Bacteria per C C	Generation Time in Minutes
0	All	223	
2	Control	1,150	52.2
4	Control	26,400	26.5
6	Control	770,000	24.7
8	Control	18,300,000	26.3
2	0.1	1,300	48.4
4	0.1	24,800	28.2
6	0.1	620,000	25.9
8	0.1	19,600,000	24.1
2	0.2	880	62.5
4	0.2	23,400	25.4
6	0.2	865,000	23.1
8	0.2	21,800,000	25.7
2	0.3	1,020	56.5
4	0.3	30,500	24.5
6	0.3	650,000	27.2
8	0.3	26,200,000	22.5
2	0.5	840	64.9
4	0.5	16,000	26.2
6	0.5	650,000	22.4
8	0.5	29,000,000	21.9
2	1.0	1,770	41.0
4	1.0	72,300	22.4
6	1.0	3,220,000	21.9
8	1.0	39,400,000	33.2

SUMMARY OF GENERATION TIMES

Time in Hours	0.0	0.1	0.2	0.3	0.5	1.0
0-2	52.2	48.4	62.5	56.5	64.9	41.0
2-4	26.5	28.2	25.4	24.5	26.2	22.4
4-6	24.7	25.9	23.1	27.2	22.4	21.9
6-8	26.3	25.2	25.7	22.5	21.9	33.2

The two tables are not in exact agreement; but of the two, the first gives the more consistent results and therefore is probably more nearly correct. A rise in the generation time may be seen in the 6-8 hour period with the higher concentration of phosphate in both experiments. This is, undoubtedly, an indication that the end of the logarithmic growth period has been reached before the 8th hour and thus the rate shows a decline. The lag period is markedly decreased by the greater concentrations of phosphate. On the whole, the effect of dipotassium phosphate seems to be to cause a production of the maximum number of organisms in a shorter time.

THE EFFECT OF VARIOUS AMOUNTS OF PEPTONE

The Committee on Standard Methods of the American Public Health Association, in their 1917 report have reduced the amount of peptone in standard mediums for water analysis from 1% to one-half per cent. An experiment was made to determine the effect of different

TABLE 16
THE EFFECT OF DIPOTASSIUM PHOSPHATE (K₂HPO₄) ON RATE OF GROWTH OF B. COMMUNIS

Time in Hours	Per Cent. K ₂ HPO ₄	Average Number Bacteria per C C	Generation Time in Minutes
0	All	462	
2	Control	1,090	97.0
4	Control	27,050	25.9
6	Control	558,000	27.4
8	Control	16,650,000	24.4
2	0.1	1,570	68.0
4	0.1	38,700	26.0
6	0.1	1,380,000	23.2
8	0.1	37,300,000	25.2
2	0.2	1,180	88.5
4	0.2	27,500	26.4
6	0.2	1,290,000	21.6
8	0.2	19,400,000	30.7
2	0.3	1,560	68.5
4	0.3	24,700	30.1
6	0.3	111,000	21.8
8	0.3	20,500,000	15.9
2	0.5	2,340	51.3
4	0.5	59,500	25.7
6	0.5	3,420,000	20.5
8	0.5	36,500,000	35.1
2	1.0	2,280	52.2
4	1.0	75,000	23.8
6	1.0	3,570,000	21.5
8	1.0	16,300,000	54.8

SUMMARY OF GENERATION TIMES

Time in Hours	0.0	0.1	0.2	0.3	0.5	1.0
0-2	97.0	68.0	88.5	68.5	51.3	52.2
2-4	25.9	26.0	26.4	30.1	25.7	23.8
4-6	27.4	23.2	21.6	21.8	20.5	21.5
6-8	24.4	25.2	30.7	15.9	35.1	54.8

amounts of peptone on the rate of growth of B. communis. The mediums were prepared by weighing out different amounts of peptone and dissolving it in distilled water, tubing it, and sterilizing it at 15 lbs. pressure for 15 minutes. The generation times of B. communis in the different concentrations of peptone were determined in the usual manner. Tables 17 and 18 show the results.

It is evident that the amount of peptone has a marked influence on the rate of growth. The total number of organisms increases with the concentration of peptone up to 5%. The two experiments are not in agreement on the 5% concentration, but the figure in the first is based on a count of over 500 colonies per plate and is probably not accurate. The figure in the second experiment, based on a very satisfactory count, is undoubtedly more nearly the true number.

TABLE 17
THE EFFECT OF CONCENTRATION OF PEPTONE ON RATE OF GROWTH OF B. COMMUNIS

Time in Hours	Per Cent. Peptone	Average Number Bacteria per C C	Generation Time in Minutes
0	0.5	175	
2	0.5	730	58.2
4	0.5	8,760	33.4
6	0.5	170,000	28.0
8	0.5	2,940,000	29.2
2	1.0	840	53.0
4	1.0	10,800	32.6
6	1.0	204,000	28.3
8	1.0	3,280,000	29.9
2	2.0	1,090	45.5
4	2.0	335,100	23.9
6	2.0	940,000	25.3
8	2.0	31,500,000	23.7
2	3.0	1,300	41.4
4	3.0	79,000	20.1
6	3.0	1,750,000	26.9
8	3.0	60,900,000	23.2
2	4.0	1,500	38.3
4	4.0	148,000	18.1
6	4.0	2,740,000	28.6
8	4.0	134,200,000	21.2
2	5.0	1,800	35.3
4	5.0	123,000	19.7
6	5.0	2,430,000	27.9
8	5.0	99,900,000	22.2

SUMMARY OF GENERATION TIMES

Time in Hours	0.5	1.0	2.0	3.0	4.0	5.0
0-2	58.2	53.0	45.5	41.4	38.3	35.3
2-4	33.4	32.6	23.9	20.1	18.1	19.7
4-6	28.0	28.3	25.3	26.9	28.6	27.9
6-8	29.2	29.9	23.7	23.2	21.2	22.2

It will be seen that the 5% peptone water gives the most rapid growth. A 2% peptone, however, gives a great increase in numbers over a 1% peptone, and where a very rapid growth is desired, this amount of peptone might be profitably used.

It is quite remarkable that the increased rate of growth with the greater concentrations of peptone is largely in the lag period, or, in other words, the organisms reach their maximum rate of growth more quickly in the concentrated peptone water.

TABLE 18
THE EFFECT OF CONCENTRATION OF PEPTONE ON RATE OF GROWTH OF B. COMMUNIS

Time in Hours	Per Cent. Peptone	Average Number Baeteria per C C	Generation Time in Minutes
0	0.5	257	
2	0.5	630	94.2
4	0.5	18,200	24.3
6	0.5	263,000	31.1
8	0.5	4,390,000	29.5
2	1.0	970	62.6
4	1.0	27,400	24.8
6	1.0	705,000	25.3
8	1.0	11,400,000	29.8
2	2.0	1,580	45.8
4	2.0	49,100	24.2
6	2.0	1,710,000	23.4
8	2.0	66,000,000	22.8
2	3.0	1,830	42.4
4	3.0	63,100	23.5
6	3.0	3,140,000	21.3
8	3.0	127,000,000	22.3
2	4.0	1,660	44.5
4	4.0	98,200	20.4
6	4.0	5,000,000	21.2
8	4.0	194,000,000	22.7
2	5.0	1,360	50.0
4	5.0	80,000	19.3
6	5.0	5,300,000	19.8
8	5.0	270,000,000	20.4

SUMMARY OF GENERATION TIMES

Time in Hours	0.5	1.0	2.0	3.0	4.0	5.0
0-2	94.2	62.6	45.8	42.4	44.5	50.0
2-4	24.3	24.8	24.2	23.5	20.4	19.3
4-6	31.1	25.3	23.4	21.5	21.2	19.8
6-8	29.5	29.8	22.8	22.3	22.7	20.4

THE EFFECT OF BEEF EXTRACT

It was thought best to try out the effect of the three-tenths per cent. beef extract recommended by the Committee on Standard Methods of the American Public Health Association, and compare the rate of growth in it with that in mediums containing five-tenths per cent. beef extract, and with another containing no beef extract. These amounts of beef extract were added to one-half per cent. peptone water, and the generation times were determined as in pre-

ceding experiments. The results (Table 19) show that three-tenths per cent. beef extract gives a much greater growth than no beef extract. A 48-hour culture was used in this experiment, which accounts for the excessive lag.

Beef extract decreases both the lag period and the generation time during the logarithmic period. Five-tenths per cent. of beef extract gives a shorter lag period than three-tenths per cent., but on the whole the difference between the rate of growth in the five-tenths per cent. and the three-tenths per cent. is not very great, as is shown by the total number of organisms.

TABLE 19
THE EFFECT OF BEEF EXTRACT ON RATE OF GROWTH OF *B. COMMUNIS*

Time in Hours	Per Cent. Beef Extract	Average Number Bacteria per C C	Generation Time in Minutes
0	All	358	
2	Control	510	235.0
4	Control	8,000	30.1
6	Control	111,000	31.6
8	Control	1,770,000	30.6
2	0.3	480	284.0
4	0.3	9,200	28.2
6	0.3	319,000	23.4
8	0.3	12,700,000	22.6
2	0.5	525	217.0
4	0.5	13,500	25.6
6	0.5	320,000	26.2
8	0.5	14,450,000	21.8

SUMMARY OF GENERATION TIMES

Time in Hours	0.0	0.3	0.5
0-2	235.0	284.0	217.0
2-4	30.1	28.2	25.6
4-6	31.6	23.4	26.2
6-8	30.6	22.6	21.8

CONCLUSIONS

After a period of lag, the rate of growth of *B. communis* at 37 C. in one-half per cent. Difco peptone is constant and at a maximum from 6-8 hours.

Subcultures, during the period of constant rate of growth, as previously shown by Barber and others, do not show lag.

Crystal violet decreases the rate of growth of *B. communis* in dilutions as great as 1:1,000,000. The effect of the dye is greatest during the lag period.

B. communis will not grow in one-half per cent. Difco peptone water containing a concentration of crystal violet greater than 1:200,000. A slight inhibition of growth is caused by a dilution of 1:1,000,000.

Brilliant green in dilutions of 1:600,000 inhibits the growth of *B. communis* in one-half per cent. Difco peptone. Inhibition disappears between dilutions of 1:6,000,000 and 1:10,000,000. A slight stimulation is noted at 1:10,000,000.

Crystal violet and brilliant green have a greater inhibitive effect on rapidly growing cultures than on old cultures. The action of dyes varies on different days.

Bile salts stimulate the growth of *B. communis* when added to one-half per cent. peptone in dilutions as high as three-tenths to five-tenths per cent. Higher concentrations of bile salts inhibit growth. One per cent. shows marked inhibition.

Increasing the concentration of peptone up to 5% stimulates the rate of growth of *B. communis*. The lag period is shortened in the greater concentrations of peptone.

Addition of dipotassium phosphate to peptone in amounts up to 1% accelerates the rate of growth of *B. communis*.

Addition of beef extract to peptone increases the rate of growth of *B. communis*; five-tenths per cent. giving slightly more rapid growth than three-tenths per cent.

The age of the culture greatly influences the rate of growth of *B. communis* during the lag period. Both the generation times and the duration of the lag are greater in old cultures. A marked increase in lag appears after 3 days.

THE FUNCTION OF FATS IN IMMUNE PROCESSES

II. PNEUMOCOCCUS AND STREPTOCOCCUS IMMUNITY

CARL C. WARDEN

From the Hygienic Laboratory, University of Michigan, Ann Arbor, Mich.

The work discussed in this paper is a continuation of earlier work¹ in which it was shown that the fats peculiar to certain bacteria and other cells constitute their specific antigens, as shown by complement fixation, antibody production in animals inoculated with the antigens, and by a new specific precipitation reaction in vitro between the fat antigens and their appropriate serum antibodies. This paper deals with the quantity of antibody produced in rabbits from inoculations with the pneumococcus and the streptococcus fat antigens and the amount of protection afforded against the organisms.

In studying the antibodies against the pneumococci obtained in previous experiments² on rabbits it was observed that while present in quantity sufficient to respond to the various tests they afforded the animals little or no protection against intravenous injections of pneumococcus in doses fatal to normal animals. For this failure there seemed to be two possible explanations. Either the fat antigens alone did not engender a protective antibody and it was necessary to take the protein of the pneumococcus into consideration, or the fat antigens had not been administered wisely, that is to say in the proper form, or state, or at proper times, or in the proper manner to induce strong protection.

The first of these explanations seemed attractive. We know that antibodies to proteins can be produced in large amounts and that they are protective, but that their specificity is somewhat limited. The studies of Gengou³ and others showed that the antibody to eggwhite was not able clearly to identify the species of animal from which the antigen was obtained, and that while casein and hemoglobin antibodies always gave precipitates with casein and hemoglobin antigens, they were not able to identify the particular source from which the protein

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¹ Jour. Infec. Dis., 1918, 22, p. 131.

² Ibid., 1918, 23, p. 133.

³ Bordet and Gay: Studies in Immunity, 1909, p. 241.

had been derived. My own studies on the fat antigens showed that their corresponding antibodies possess high specificity, so high in fact as to distinguish clearly between Types I, II and III of the pneumococcus. From these facts it seemed as though the antibodies derived from an attack of an infectious disease or from active immunization with bacteria might depend on the protein fraction of the antigen for their enduring and protective qualities and on the fat fraction for their specificity. Since pneumococcus antibody in whatever way hitherto obtained is invariably small in quantity, of low protective value and yet of high specificity it would appear as if there were some fault in the pneumococcus protein. It was thought then that if there should be substituted for the pneumococcus protein a protein derived from a micro-organism known to produce antibodies of long continuance and high protective value, such for instance as protein from the typhoid bacillus, and if to that protein there should be "grafted," so to speak, the specific fat antigen of the pneumococcus it might be possible with such a combination to produce in animals antibodies having the combined qualities of the respective antigens, namely, protective and specific factors. To test this assumption the following experiments were carried out.

Exper. 1.—A protein was obtained from the defatted typhoid bacillary substance of Vaughan by extraction with distilled water containing a few drops of chloroform at 37 C. for a week, centrifugating the undissolved residue and evaporation of the supernatant and slightly straw-colored liquid to dryness at 20 C. under an air blast. The resulting brittle substance was easily powdered and almost wholly soluble in water and physiologic salt solution. Such solutions contained no coagulable protein but gave strong protein reactions with biuret and Hopkins-Cole reagents, and contained approximately 0.002% free amino-acids as determined by the van Slyke method. This substance was toxic for rabbits, being invariably fatal within 4-8 hours in doses of 0.1 mg. The dose adopted for immunizing purposes was 0.05 mg. which could be repeated with safety as often as every other day. Type I pneumococcus was used, the invariably fatal dose of which for rabbits of 1,800 to 2,000 gm. was found to be 0.05 cc of a 24-hour glucose broth culture, thoroughly shaken. This dose produced death from septicemia within 72 hours.

The pneumococcus artificial general antigen² was used for grafting on the protein for purposes of immunization. This consisted of the sodium esters of the fatty acids common to Types I, II and III of the pneumococcus and was administered intravenously dissolved in salt solution.

Two series of rabbits averaging 1,800 gm. weight were inoculated in duplicate in the following manner: Four rabbits received 4 intravenous inoculations at intervals of 48 hours of 0.05 mg. typhoid protein in 2 cc salt solution. Four received similarly spaced inoculations of 5 mg. of the pneumococcus fat antigen dissolved in 2 cc salt solution; and 4 received similar inoculations of the same quantity of the antigen to which solution there was added 0.05 mg. of typhoid protein.

The rabbits were bled from the heart on the 8th day following the last inoculation and the serums tested for antibodies.

Briefly, the results of the test were as follows: The rabbits injected with typhoid protein gave serums precipitating with the protein antigen solution, 1:1,000, and to some extent also with tubercle bacillary protein. Normal horse serum also gave slight precipitates with these antigens whereas pooled normal rabbit serum did not. The serum from the rabbits injected with the pneumococcus fat antigen agglutinated suspensions of pneumococcus type I in equal volumes, and gave precipitates in doses of 0.08 cc with 0.5 cc volumes of the antigen solution containing 0.2 mg. of antigen. The serum from the rabbits injected with the mixed antigens showed antibodies precipitating with the typhoid and the fat antigens severally and combined, but indicating no marked avidity for the combined antigens.

Two days following the bleeding, that is 10 days after the last inoculation, the rabbits received intravenously the invariably fatal dose of pneumococcus culture. All died within the prescribed time of 72 hours, those having received the protein being the first to succumb.

Further trials of the combined antigens on fresh animals showed that in some the time of illness was prolonged and that joints became involved, and it was thought that such instances might show a slight degree of protection probably due to the protein antibody, but the idea was abandoned after similar conditions were observed occasionally to obtain in rabbits injected with fat antigen alone.

From this experiment it was apparent that the assumption on which it was based was erroneous.

It was then determined to ascertain whether the protective power of antibody could be augmented by varying the pneumococcus fat antigen. The fats composing the antigen are of such character that their alkali salts are easily soluble in water and in physiologic salt solution, forming solutions either quite clear or faintly opalescent in the strength employed for inoculation which undergo hydrolysis slowly. The microscope shows no particles in suspension. With the idea of giving greater "surface" to the solutions, or, in other words, of making the antigen a suspension of fairly uniform sized particles more stable and colloidal in character there were presented several expedients, one of which was to produce the desired surface by the addition of suitable amounts of a kation, such for instance as Ca, to the solution of the sodium salts of the antigen, while another was to substitute for the Na another metal which would give fatty salts of less solubility and more easily aggregated by a bivalent kation. The latter idea was chosen and an antigen was made of the lithium salts of the appropriate fatty acids. These salts when dissolved or suspended in Ringer-Locke solution of 0.025% strength gave dense white suspensions, the particles in which, crystalline in character, were visible with a $\times 8$ lens.

Exper. 2.—Four rabbits averaging 1,800-2,000 gm. weight received intravenously at 48-hour intervals 0.5 mg. of the pneumococcus lithium antigen in 2 cc Locke solution. Five days after the last of the injections 2 of the rabbits were bled and their serums shown to contain antibodies by the tests of agglutination of germ suspensions and precipitation with antigen solution. On the 8th day following the last injections the 4 animals together with 2 normal controls were inoculated intravenously with the fatal dose of pneumococcus (Type I) culture. The controls died within 72 hours, 2 of the inoculated animals died within the same period and a third in 96 hours. One rabbit was made ill but recovered.

A second series of 4 rabbits of the same average weight received 6 intravenous inoculations at 48-hour intervals of 0.5 mg. of the lithium antigen in 2 cc of Locke solution. Two days after the last injection, without preliminary bleeding for purpose of testing the serums, the inoculated rabbits and the usual controls were injected intravenously with twice the fatal dose of pneumococcus Type I, that is, 0.1 cc of a 24-hour glucose-broth culture diluted to 1 cc with salt solution. The control animals died on the 3rd day, 2 of the injected rabbits died on the 5th day, while the 2 remaining recovered.

While this series was in progress 4 fresh normal rabbits of even average weight received at the same time with the preceding controls each a single fatal dose of the same culture, which was followed, after 1 hour, by intravenous inoculation of 0.5 mg. of the lithium antigen in 2 cc of Locke solution. This antigen injection was repeated in each animal on the 2 following days, making 3 injections in all. Of these rabbits 2 recovered and 2 died on the 5th day.

It appeared from this experiment that the antibody afforded a considerable measure of protection. It was thought, however, that the antigen would be distinctly improved could it be brought into a more stable colloidal form approaching as closely as possible the state in which the fats might be supposed to exist in the surfaces of the cocci. Previous experience with colloidal cholesterol² led to the belief that the state of the fats desired might be approximated through the agency of this substance. Accordingly the pneumococcus fats were converted into the cholesterol esters of the fatty acids⁴ and the general type of antigen prepared from these materials. The antigen made from the cholesterol esters is nearly solid at 20 C., and wholly immiscible with water. It is soluble in ether and in hot acetone, from which solutions it forms very stable colloidal solutions on the addition of several volumes of distilled water and evaporation of the solvent. Such solutions of 1:1,000 are white by reflected light, and blue-white, faintly translucent and opalescent by transmitted light. A very few of the larger particles are visible as dots under high microscopic power. The addition of electrolytes causes aggregation and increase in the

⁴ For methods see Lewkowitsch, *Chem. Tech. of Oils, Fats and Waxes*, 1913, p. 66 and p. 271.

size of the particles, and the whole of the esters may be flocculated out by suitable concentrations of kation such as Ca. Calcium chlorid added to the colloid in such amount as to make the solution 1:10 M. produces aggregates visible with a $\times 8$ lens. Concentration of 1:200-1:100 M. gives rise to particles best seen in hanging drop under $\frac{1}{12}$ oil immersion varying in size from 0.5-6 mikrons, the majority being about the size of ordinary cocci. They are round, slightly refractile, have lively brownian movement and the resemblance to cocci is striking. Diplococcal forms are not uncommon and occasionally short chains of 3 or 4 are seen. The colloid made from solution in ether is not so homogeneous nor so stable, but contains even greater variety of forms, many of them capsulated. The colloid made from acetone solution remains apparently unchanged for months at room temperature, can be sterilized by boiling without injury and is the more satisfactory form to work with.

Exper. 3.—Eight rabbits of 1,800 gm. average weight received 4 intravenous injections at 48-hour intervals of 0.5 mg. of the pneumococcus cholesterol colloid antigen in 2 cc Locke solution. Two days following the last of the injections 4 rabbits were given twice the fatal dose of pneumococcus I culture intravenously. These animals recovered. Four days after the last injection 2 of the remaining rabbits received intravenously 3 times the fatal dose of pneumococcus culture. One died in 3 days and one recovered. The 2 remaining rabbits received at the same time as the preceding pair 4 times the fatal dose of culture. Both animals died within 72 hours. Synchronous with the injection of 2 control rabbits 4 fresh rabbits of the same average weight received also one fatal dose of culture and, after 1 hour, 0.5 mg. of pneumococcus cholesterol colloid antigen the injections of antigen being repeated daily until 3 doses had been given. The control animals died within the specified time, the 4 inoculated animals recovered. Eight days after the last inoculations the surviving rabbits were bled and all serums were found to give strong precipitation tests with the antigen mentioned under *Exper. 1.*

From this experiment it was apparent that the antibody induced by the cholesterol colloidal antigen afforded greater protection to the rabbits than those consisting of the Na and Li esters. It is believed that further improvement can be expected as one learns how and in what state the antigen is best administered. It is evident that in an aqueous colloidal solution of an antigen* composed of several fat ingredients some of them will be aggregated to the desired proportions by certain concentrations of electrolyte, whereas others will not; in other words, all the ingredients will not be in the same state, so that the probable effect on the animal will be a retardation in the production of certain elements of the antibody. But at the same time it becomes

apparent that in all probability the fluids and cells of the injected animal respond better to certain fats than to others, and from our observations up to the present time it appears that the more stable in physicochemical characters the antigenic fats, the more stable and protective the antibody engendered. The aliphatic series of normal acids from acetic at the lowest end to clupanodonic at the other includes all the antigenic fats found in the organisms and cells thus far studied. At either end of the series are soluble and unstable acids while in the middle are found those most stable and therefore most readily influenced by electrolytes and other factors which regulate aggregation or dispersion. As the antigenic fats of bacterial cells approach either extreme the production of enduring and protective antibody becomes apparently more difficult, while an organism whose fats occupy middle ground develops strong antibody. The typhoid group of organisms well represents the stable type and the pneumococcus group one of the unstable.

The conditions of the foregoing experiments were purposely made somewhat severe in order to place the pneumococcus antigen under strict test. Type I pneumococcus was chosen because of the regularity of infectiousness for rabbits, the brevity of the course of the infection and the smallness of the fatal dose. The "blanket" pneumococcus antigen was selected in order to determine whether it would afford protection against the most infectious of the types, although it does not strictly represent the true antigen for Type I. It will be recalled² that while the general antigen covers Types I, II and III, its ingredients form the average fat antigen content of all 3 and is not the exact antigen for Type I, but is the exact one for Type II, the 3 types having been shown to contain the same fat substances only in slightly different proportions. From the results of the experiments it seems justifiable to infer that if the blanket antigen affords a degree of protection, as it has been shown to do, against the most virulent member of the group it will induce protection against the other less virulent members.

While the results of the experiment with bacterial protein are of course inconclusive, nevertheless, the impression gained from the work up to the present moment has become progressively stronger that protein immunization and specific cell immunization may be two quite distinct processes, and that the rôle of protein in cell immunity is probably so slight as to be almost if not wholly negligible. With the

protein immune process occur the factors of sensitization, toxicity, specificity for type only and not for species, while with fat immunization there is no sensitization and no toxicity, but marked species specificity. Rabbits immunized with fat antigens never show sensitization on reinjection after a lapse of time, nor do toxic symptoms occur except in the manner of anaphylaxis which may be induced by the "surface" of the antigen suspensions in the same way as by the surface of agar, starch or inulin suspensions. In the preceding paper⁴ there were mentioned instances of undoubted sensitization in rabbits following reinjection with fats. The sudden deaths in these animals may have been and probably were due to anaphylaxis from surface action.

THE STREPTOCOCCUS ANTIGEN

The cultures of streptococcus used consisted of 6 strains each of *S. viridans* and *S. hemolyticus*, all of which had been isolated from cases of acute infections in men a short time prior to this study. The fats of the combined strains of organisms were obtained in a manner identical with that employed in the case of the pneumococcus antigen,² examined, and from the data acquired there was prepared an artificial streptococcus antigen of the general type.

By the methods of precipitation and agglutination employed as before described, the antigen was tested against supposed antistreptococcus serums prepared by the commercial houses and purchased in the market. Briefly, one of two conclusions was to be drawn from the tests. Either the antigen was faulty or the serums contained no antibody. Inasmuch as the serums were found to possess no powers of agglutinating streptococci beyond that shown by normal horse serum or physiologic salt solution, and gave no precipitates with the antigen, and as the antigen represented the average of several close fat determinations it was concluded that the fault did not lie with the antigen. If, then, it could be shown that injections of the fat antigen into rabbits would produce antibody with power of agglutination over the cocci and of precipitation of the antigen the conclusion that the antigen had been approximated would be justified. Accordingly, the injections were carried out.

Exper. 4.—As representative of the method employed the protocol of one series of animals is noted here. Four rabbits of 2,000 gm. average weight received 5 intravenous inoculations of 0.5 mg. of the streptococcus cholesterol colloid antigen in 2 cc of Locke solution. Three days after the last injection

the rabbits were bled and their serums tested, two for agglutinating power and all for precipitation.

The suspension of streptococcus was made as follows: Twenty-four-hour glucose-broth cultures of the various strains of streptococci were combined and centrifugated, the clear broth pipetted off and the residue suspended in 0.6% salt solution. This suspension was then thoroughly shaken and again centrifugated for a short time at moderate speed to remove clumps. The resulting emulsion was fairly thick and homogeneous. The serum dilutions were 1:10, 1:50 and 1:100. The test tubes after mixing were placed in the icebox for 8 hours.

Rabbit 1 serum showed complete agglutination in dilution of 1:10, partial in 1:50 and slight in 1:100.

Rabbit 2 serum showed partial agglutination in dilution in 1:10, less in 1:50, and none in 1:100.

Normal rabbit serum produced no agglutination in the same dilutions. The control suspension was not agglutinated.

The antigen used consisted of an alcoholic solution of the sodium salts of the fatty acids of the streptococcus in such strength that 1 cc contained 2 mg. of the salts. To 1 cc of this solution there was added 0.6 cc of a 1% alcoholic solution of cholesterol. The dose of this completed antigen, 0.1 cc, therefore contained 0.125 mg. of fat and 0.375 mg. of cholesterol. The tubes were placed in the icebox for 6-8 hours.

TABLE 1
PRECIPITATION

With 0.5 cc Salt Solution plus 0.1 cc Antigen

Serum 1, 0.08 cc = partial precipitation	Serum 3, 0.24 cc = complete precipitation
Serum 1, 0.24 cc = complete precipitation	Serum 4, 0.08 cc = partial precipitation
Serum 2, 0.08 cc = complete precipitation	Serum 4, 0.24 cc = complete precipitation
Serum 2, 0.24 cc = partial precipitation	Salt solution, 0.08 cc = no precipitation
Serum 3, 0.08 cc = complete precipitation	Nor. rabbit serum, 0.24 cc = no precipitation

From this experiment it was concluded that antibodies against the streptococcus were produced in the serums of the injected animals.

Owing to difficulties the protective power of the antibodies against infection by streptococcus was not determined in rabbits. The strains of organisms used were found to possess low virulence. Large doses were necessary to infect, no uniform dose produced like degrees of infection and the infection itself was of the chronic type, many of the rabbits recovering. By means of the precipitation test it was observed that the serums of some rabbits apparently contain a slight amount of antibody against the streptococcus, but no serum was found to hold any amount comparable to that in the serums of the inoculated animals.

In order to test whether rabbits having no streptococcus antibody, or slight amounts only, would develop it following inoculations of the antigen in another form and in a different manner, 3 selected rabbits were given 4 subcutaneous injections, 2 days apart, of 0.5 mg. of the

streptococcus antigen composed of the lithium salts. On the 3rd day following the last injection the rabbits were bled. Table 2 shows the presence of antibodies in greater or less amount.

TABLE 2
PRECIPITATION

With 0.5 c c of 0.6% Salt Solution plus 0.1 c c Antigen

Serum 1, 0.08 c c = no precipitation	Serum 3, 0.08 c c = partial precipitation
Serum 1, 0.16 c c = partial precipitation	Serum 3, 0.16 c c = partial precipitation
Serum 1, 0.24 c c = complete precipitation	Serum 3, 0.24 c c = partial precipitation
Serum 2, 0.08 c c = partial precipitation	Serum nor. rabbit, 0.24 c c = no precipitation
Serum 2, 0.16 c c = partial precipitation	Salt solution, 0.08 c c = no precipitation
Serum 2, 0.24 c c = complete precipitation	(Icebox 8 hours)

The availability of subcutaneous inoculations of fat antigens for the production of immunity in man is illustrated by the following experiment.

Exper. 5.—Four men between the ages of 20 and 40 years were bled and their serums shown by complement fixation, agglutination and precipitation tests to contain no antibodies against, respectively, the typhoid bacillus, the pneumococcus, the streptococcus and the treponema pallida.

1. Received 3 subcutaneous injections of 5 mg. of pneumococcus cholesterol colloid antigen in 2 c c salt solution containing CaCl_2 1:100 M. at intervals of 2 days.

2. Received 4 similar injections of a like amount of streptococcus colloid antigen at intervals of 4 days.

3. Received 4 similar injections of typhoid antigen.

4. Received 4 similar injections of syphilis antigen.

Five days after the last injection the men were bled again. Their serums gave precipitates with their respective antigens but not with crossed antigens. The serum of the man last mentioned also gave a positive syphilitic complement fixation test. The serum of the 3rd man agglutinated suspensions of *B. typhosus*, parat. A, parat. B. and *B. coli* in dilutions of 1:200.

Granted that animals develop antibodies against certain combinations of fats which represent the antigenic factors in cells it should be possible to induce antibody in rabbits by injection of a single, isolated fat, in which case the interaction of antigen and antibody in precipitation would afford a biologic test for the fat in question.

Exper. 6.—Two rabbits weighing approximately 2,000 gm. were bled from the heart and their serums tested for precipitation with an antigen composed of an alcoholic solution of sodium oleate containing 2 mg. of the salt in 1 c c, to which was added a 1% solution of cholesterol (alcoholic) in the proportion of 0.6 c c for each 1 c c of the oleate solution. The serums caused no precipitation with 0.1 c c of the antigen in 0.5 c c of salt solution in doses as high as 0.32 c c. The salt of oleic acid was chosen as being one of the most difficult in the series with which to produce antibody owing to its solu-

bility and perhaps also to its slightly unsaturated character. Another reason for the choice was because of its common occurrence as such or in the form of the glycerid in animal tissues.

The 2 rabbits then received 4 intravenous injections of 0.5 mg. of lithium oleate in 2 cc of Ringer solution, the second injection being given after an interval of 4 days and the following injections at intervals of 2 days. Two days after the last inoculation the rabbits were bled and their serums tested with the sodium oleate antigen as follows.

TABLE 3
PRECIPITATION TEST

With 0.5 cc of 0.6% Salt Solution Plus 0.1 cc Antigen,

Serum 1, 0.12 cc = partial precipitation	Serum 2, 0.08 cc = partial precipitation
Serum 1, 0.20 cc = complete precipitation	Serum 2, 0.20 cc = partial precipitation
Serum 1, 0.32 cc = complete precipitation	Serum 2, 0.32 cc = partial precipitation
Serum nor. rabbit, 0.32 cc = no precipitation	Salt solution, 0.08 cc = no precipitation

(Icebox 6-8 hours)

One week later the 2 rabbits received 2 intravenous injections, with an interval of 2 days, of 2 cc of cholesterol oleate colloid antigen containing 0.5 mg. of the ester. Three days after the last injection the rabbits were again bled. Their serums showed practically no increase in antibody. Without further injections the animals were bled again after the expiration of 1 week. The results of the precipitation test with the sodium oleate antigen, together with similar antigens of sodium linoleate, and sodium palmitate, acids respectively above and below oleic, are shown in Table 4.

TABLE 4
PRECIPITATION TEST

With 0.5 cc Salt Solution Plus 0.1 cc Oleic Antigen; 0.1 cc Linoleate Antigen; Palmitate Antigen,

Serum 1, 0.08 cc = complete precipitation	Serum 2, 0.24 cc = complete precipitation
Serum 1, 0.24 cc = complete precipitation	Serum 1, 0.24 cc = complete precipitation
Serum 2, 0.08 cc = complete precipitation	Nor. rabbit serum, 0.24 cc = no precipitation
Serum 2, 0.24 cc = complete precipitation	Antistrep. ser., 0.24 cc = no precipitation

No precipitations with palmitate or linoleate antigens.

From this experiment one may tentatively conclude that rabbits may be immunized against an isolated fat, and that their serums will react with precipitation to the fat in the proper antigen, but not to fats closely related but with different characters and molecular weights. It will be observed in the table that a pneumococcus antiserum gave a precipitate with the sodium oleate antigen. This phenomenon might be expected since oleic acid is an important constituent of the pneumococcus fats, and is quite comparable if not identical in causation with that of group agglutination and complement fixation. It has been a matter of occasional observation in the course of this work that when one fat is a prominent ingredient of 2 antigens the antisera to those antigens will give partial precipitation with the related antigen

but not with antigens in which the fat plays a minor part. Certain unfinished experiments, however, seem to indicate that an immune serum against an antigen composed of several fats will give precipitates more or less complete with the individual fats composing it. Thus it has been observed that antityphoid serum will precipitate not only with the typhoid antigen,² but also with solutions of each of its several components, probably because the latter exist almost in equal parts in the combination.

The apparent power of an immune serum to precipitate with the individual components of a fat antigen complex led to the inquiry whether it were possible by such means to approximate qualitatively the fatty constituents of all the fatty antigens in use, in which case the results would be a check on the chemical analysis of the cells. This has not been possible in all instances thus far. Where a constituent of an antigen exists in small amount only, an immune serum, while precipitating with the fat as part of the antigen complex, does not precipitate with the individual fat in the ordinary concentrations as used in antigen-salt solution-serum mixtures. It appears that there is required different proportions of electrolyte, fat and serum. These proportions have not yet been worked out, but from the inquiry some interesting suggestions have risen. For instance, working with pooled syphilitic serums the results of precipitation with individual fats gave qualitative results corresponding very closely with the analysis of the syphilitic antigen; and while an analysis of human red cells has not been attempted a qualitative suggestion was obtained from immune rabbit serum as shown in Table 5.

TABLE 5
RESULTS OF PRECIPITATION WITH INDIVIDUAL FATS

Fat	Antigens, 0.75 c c	Alcoholic 0.6% Salt Solution	Solution in	Total Quantity of Mixtures 0.75 c c	
				Normal Rabbit Serum, 0.2 c c	Rabbit-Human Serum, 0.2 c c
Sodium Caprate	0.06 gm.	No precipitation	No precipitation	No precipitation	Partial precipitation
Sodium Laurate	0.06 gm.	No precipitation	No precipitation	No precipitation	Partial precipitation
Sodium Myristate	0.06 gm.	No precipitation	Partial precipitation	Complete precipitation	Complete precipitation
Sodium Palmitate	0.05 gm.	No precipitation	No precipitation	Partial precipitation	Partial precipitation
Sodium Stearate	0.06 gm.	No precipitation	No precipitation	Partial precipitation	Partial precipitation
Sodium Cerotate	0.07 gm.	No precipitation	No precipitation	No precipitation	No precipitation
Sodium Melyssate	0.07 gm.	No precipitation	No precipitation	Complete precipitation	Complete precipitation
Sodium Oleate	0.07 gm.	No precipitation	No precipitation	Complete precipitation	Complete precipitation
Sodium Linolate	0.07 gm.	No precipitation	No precipitation	Complete precipitation	Complete precipitation
Sodium Clupanodinic	0.07 gm.	No precipitation	No precipitation	Complete precipitation	Complete precipitation

(Icebox 8 hours.)

These reactions suggest a highly complex composition for human red cells which no doubt is not far from the truth since they of all animal cells must be "immune" to the widest range of fats.

The reaction of precipitation, or agglutination, between immune serums and fats must be regarded as largely tentative. It requires the balance of ingredients and delicacy of manipulation as correct as in complement fixation, on which indeed it is in part founded, and the result of a large number of experiments must be observed before final deductions may be drawn. Particularly troublesome are spontaneous precipitations of antigen and salt solution controls which occur when proportions or measurements are incorrect. Some individual fat controls demand stabilization with a dispersive colloid such, for instance, as 0.01 c c or 0.02 c c of normal rabbit or human serum, a procedure which is entirely permissible.

The facts brought out in the work on the functions of antigenic fats in immunity lead one to believe that such antigens are destined to play an important part not only in active immunization of animals and man as a prophylactic measure, but also in the treatment of infections. They have to commend them their purity, the dosage by weight, the absence of toxicity, the ease and safety of either subcutaneous or intravenous administration.

This form of treatment would appear to be particularly applicable to the types of infections of acute and often fatal character and of brief duration where antibody production is invariably slight or absent altogether, and where toxemia is the dominant symptom, such types being represented by pneumococcus, streptococcus and meningococcus infections. In infections also of a more prolonged course such as typhoid and paratyphoid fevers, even when antibody is known to be present in the serum, as shown by agglutination tests, at the same time with the antigenic micro-organism, the fat antigen would be presumed to be beneficial both by increasing the antibody production and by furnishing a nidus round which the antigen-antibody aggregate might form and lead to the absorption of complement.

THE FORMATION OF CONGLUTINATION THROMBI IN THE LIVER OF DOGS AFTER INJECTIONS OF WITTE'S PEPTONE

J. P. SIMONDS

From the Department of Pathology of Northwestern University Medical School, Chicago

In the course of studies on the ereptase content of the blood and organs of dogs, an attempt was made to increase the amount of this ferment by repeated injections of Witte's peptone. The injections were given into the saphenous vein at intervals of 2-4 days. A first dose of 1 gm. for an average sized dog was used. The succeeding doses were increased until several animals received 10 gm. of peptone intravenously at one time. The injections were made fairly slowly. The reaction following the first dose was quite marked—discharge of feces and urine, sometimes vomiting, and marked depression, but no definite evidence of the pronounced bronchoconstriction as observed by Hirschfelder¹ in guinea-pigs which had been injected with peptone. The reactions from the subsequent larger doses were either absent or less severe. The animals were killed by chloroforming immediately after the last injection or at the end of 8 and 15 days. For purposes of comparison a number of dogs that died as a result of acute peptone shock following a first injection are included in this series.

The liver in all the animals showed characteristic microscopic changes. In the dogs dying in acute peptone shock the changes closely resemble the condition seen in marked passive hyperemia. There is an excess of blood in the central veins and sinusoids and in the branches of the portal vein in Glisson's capsule. The sublobular and other branches of the hepatic vein are not distended. In many places the sinusoids show unusually wide, localized, circular or oval dilatations.

In the livers of dogs killed after 8 and 15 days, respectively, solid plugs are seen occupying round or oval spaces where the sinusoids are distended to 2-4 times their normal width. The number of these plugs does not differ greatly in different livers, and range from 1-6 in a single (low power) field of the microscope. They occur in all parts of the lobule but are most numerous in the midzonal and peripheral portions. They are not found in the branches of the portal vein. The hepatic cells adjacent to the plugs appear normal. No other organs show such masses in any of their blood vessels.

These intrasinusoidal plugs are composed of material that is partly granular and partly homogeneous or hyaline, and most of them contain granules of reddish brown pigment. They take the eosin stain. Stains for fibrin show

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¹ Jour. Exper. Med., 1910, 12, p. 586.

that this is present in small amount in the 8-day specimens. The plugs in some instances do not fill the spaces in which they lie. In the 15-day specimens, and to a less extent in the 8-day specimens, these masses have been invaded by large mononuclear or transitional ("endothelial") leukocytes, many of which contain granules of pigment. No genetic relation can be established between these cells and the adjacent sinusoidal endothelium which appears normal. This may be due to the late stage at which the specimens were observed (8-15 days).

In attempting to explain the origin of these intrasinusoidal plugs, two possibilities suggest themselves. In the first place, they may be due to a localized thrombosis of the sinusoids, such as is not infrequently seen in cases of passive hyperemia of the liver. Nolf² has shown that if a solution of peptone is injected slowly, the coagulability of the blood is greatly increased. He has employed repeated injections of peptone to prevent hemorrhage in typhoid fever, and in the treatment of hemophilia and related conditions. Pozerski and Pozerska³ found that the so-called immunity to the anticoagulant action of peptone after injections is more lasting than the resistance to its depressor action. Nolf⁴ has explained the occurrence of leukopenia after injections of peptone on the basis of damage to the endothelium of the hepatic capillaries thus rendering them more sticky for leukocytes. There is a possibility, therefore, that the coagulability of the blood of the animals in these experiments was increased by the injections of the peptone. Unfortunately no studies were made to clear up this point at the time, because the primary object of the experiments was a study of ferments and the changes in the liver were only discovered during the routine microscopic examination of the tissues of animals used in experiments in this laboratory.

If such increased coagulability did exist, thrombosis in the intra-lobular capillaries of the liver would be most likely to occur in the locations in which these plugs have been found, namely, in the circular or oval dilatations of the sinusoids where the blood flow would be slowest and any damage to the endothelium probably the greatest.

On the other hand, the formation of plugs from conglutinated red blood corpuscles must be considered. Such thrombi have been observed by Flexner⁵ after the injection of certain toxalbumins, such as abrin and ricin, and by Pearce⁶ and by Karsner and Aub⁷ after

² *Compt. rend. Soc. de biol.*, 1916, 79, p. 648.

³ *Ann. de l'Inst. Pasteur*, 1913, 27, p. 120.

⁴ *Arch. Internat. de Physiol.*, 1904, 1, p. 242.

⁵ *Jour. Med. Research*, 1902, 8, p. 316.

⁶ *Jour. Med. Research*, 1904, 12, p. 329.

⁷ *Jour. Med. Research*, 1913, 28, p. 377.

injections of hemagglutinating serum. The masses of agglutinated erythrocytes produced by the toxalbumin and by the serum were formed in the circulating blood and carried to the liver. Here they lodged as a rule in the mid-zonal region of the lobule, which, as pointed out by Opie,⁸ is the location at which minute emboli in the portal vein usually come to rest. Pearce also observed similar masses in the vessels of the kidneys of animals injected with hemagglutina-tive serums.

In the condition here described, the mechanism of conglutination of the red blood cells was probably somewhat different. There is no evidence that Witte's peptone causes agglutination of the red cells of the dog as do abrin and ricin. After injections of peptone there is an obstruction to the outflow of blood from the liver. The central veins, sinusoids and distributing branches of the portal vein are distended with blood. At the same time there is a marked loss of fluid from the blood in the liver as shown by numerous experiments on lymph formation by Heidenhain,⁹ Starling,¹⁰ Yanagawa¹¹ and others.

The stagnation of blood in the dilated sinusoids, with the escape of much of its fluid into the lymph stream, leads to the formation of densely packed aggregations of red blood corpuscles. This occurs most readily in the circular or oval dilatations previously mentioned. Such groups of corpuscles, closely crowded together with very little intervening fluid, do not readily break up when the obstruction to outflow is relieved and the circulation in the liver restored to normal. Instead, they become fused into solid masses in which the individual corpuscles lose their identity.

The hepatic epithelium adjacent to the intrasinusoidal plugs shows no definite evidence of either hyaline necrosis as observed by Pearce⁶ and by Karsner and Aub⁷ after injections of hemagglutinating serum, or of the granular necrosis noted by Mallory¹² after injections of lycopodium spores into the portal vein. It cannot now be stated whether the absence of such necrosis following the injections of peptone here described is due to the late stage at which the examinations were made, complete regeneration having taken place in the meantime; or to differences in the extent of the occlusion of the sinusoids; or to the absence of cytotoxic amboceptor from the con-

⁸ Jour. Med. Research, 1904, 12, p. 147.

⁹ Arch. ges. Physiol., 1891, 49, p. 209.

¹⁰ Jour. Physiol., 1894, 16, p. 224.

¹¹ Jour. Pharm. and Exper. Therap., 1917, 9, p. 75.

¹² Jour. Med. Research, 1901, 6, p. 264.

glutinated corpuscles, which Pearce⁶ and Karsner and Aub⁷ believed was liberated from the thrombi produced as a result of the injection of hemagglutinating serums.

SUMMARY

In dogs dead of acute peptone shock, the microscopic appearance of the liver resembles very closely that of marked passive hyperemia.

In the liver of dogs which have received repeated injections of Witte's peptone intravenously, there are found quite constantly in many of the sinusoids solid plugs of more or less homogeneous material.

In the stages of the process here studied (8 and 15 days after the last injection of the peptone), no changes were observed in the adjacent liver cells or endothelium.

It seems probable that these intrasinusoidal masses were formed *in situ* from agglutination of red blood corpuscles in localized areas of unresolved stasis due to obstruction to the outflow of blood from the liver and to the great loss of fluid from the blood in the liver in peptone poisoning.

STUDIES ON BACTERIAL NUTRITION

THE UTILIZATION OF NITROGENOUS COMPOUNDS OF DEFINITE CHEMICAL COMPOSITION

STEWART A. KOSER AND LEO F. RETTGER

*From the Sheffield Laboratory of Bacteriology and Hygiene, Yale University, New Haven,
Conn.*

INTRODUCTION AND REVIEW OF LITERATURE

The recent studies on bacterial nutrition have emphasized the important part played by the simple nitrogenous compounds in furnishing a readily available source of nitrogen. Some knowledge of the rôle played by these substances has been attained as a result of a number of very important and interesting contributions to this subject.

The work of Bainbridge (1911) demonstrated that purified unaltered animal proteins, when supplied as the sole source of nitrogen, failed to furnish the necessary conditions for the development of certain aerobic and facultative anaerobic bacteria. This was substantiated by Sperry and Rettger (1915) who showed, in addition, that the putrefactive anaerobes were unable to attack native proteins and that pure vegetable proteins, as edestin, exhibited the same resistance to direct bacterial attack. They considered this resistance to be due to the complex construction of the protein molecule, which rendered it unfit for immediate utilization.

Rettger, Berman, and Sturges (1916) found that coagulated albumin shows the same resistance to the direct action of bacteria as do the unchanged native proteins. They also demonstrated that proteose and peptones are not attacked, or at least very slowly, by the gelatin nonliquefying species. On the other hand, the organisms elaborating a proteolytic enzyme accomplished the destruction of these substances quite readily.

Attention will be directed in this paper to the amino-acids and other simple nitrogenous compounds, for it is to these substances that one must turn to find sources of immediately available nitrogen.

Several investigators have obtained striking results by employing protein-free mediums composed of a mixture of the products of enzymatic protein cleavage. Dalimier and Lancereaux (1913) made use of a product known commercially as opsine. They succeeded in cultivating not only the commoner saprophytes but also many of the more exacting pathogens, as *B. typhosus*, *B. diphtheriae*, *B. anthracis*, *B. tetani*, *B. tuberculosis* (human, bovine and avian), the pneumococcus, meningococcus, and gonococcus.

Robinson and Rettger (1918), in a more extensive study of opsine, were able to cultivate the obligate anaerobes, the Welch bacillus and many other exacting pathogens, in addition to the above-mentioned organisms. They also found that better results were obtained with the enzymatic digestion products of pro-

tein, opsin, than with the acid hydrolysis products of edestin, lactalbumin, or casein.

However, when one or more chemically definite nitrogenous compounds have been substituted for the above heterogeneous mixtures, the results have not been so promising. Uschinsky's medium (Uschinsky 1893 a, b) and Fränkel's modification (1894) support a good growth with many of the commoner saprophytes and a few of the less exacting pathogens, but both fall far short of constituting an ideal definite chemical culture medium.

Numerous studies have been made of the ability of various species of bacteria to utilize chemically-definite nitrogenous foodstuffs when supplied as the only source of nitrogen. Thus, the tubercle bacillus has been studied by Proskauer and Beck (1894), Frouin (1912), Armand-Delille et al. (1913), Tiffeneau and Marie (1912), and by Magoon (1916), all of whom have reported successful cultivation on various mediums of a known chemical structure. Bielecki (1911) investigated the development of the anthrax bacillus in solutions of amino-acids. Hadley (1907) claimed to have succeeded in obtaining growth and toxin production by *B. diphtheriae* in a "synthetic" medium. Dolt (1908) prepared several simple "synthetic" mediums for growing the colon bacillus and recommends two of these as suitable for water analysis. Clark and Lubs (1917) recently recommended a medium consisting of anhydrous Na_2HPO_4 0.7%; KH-phthalate, 0.2%; aspartic acid, 0.1%, and dextrose, 0.4%, for the differentiation of the colon-aerogenes group. Both *B. coli* and *B. aerogenes* grew well and no diminution in the growth or vigor of fermentation was observed on a number of successive transfers through the above medium.

Zunz and György (1916) studied the influence of proteoses, peptids, amino-acids, purins, urea, and extractive bodies on the development and indol production by *B. coli* and *B. typhosus*. Sasaki (1912 a) found that *B. coli* was able to effect a splitting of the dipeptids glycyl-tyrosin and glycyl-glycin. Later (1912 b, c) he extended these observations and obtained the same results with various members of the colon-typhoid-dysentery group, *Micrococcus tetragenus* and 12 species of gelatin liquefiers. In another publication (1914) he demonstrated the production of p-oxyphenylethylamin from tyrosin by *B. coli* in a chemically-definite medium.

Jordan (1899) employed a number of "synthetic" mediums for the purpose of studying the production of fluorescent pigment by bacteria. Blanchetière (1917) investigated the mode and conditions governing the attack on asparagin by *Bacillus fluorescens liquefaciens*. Franzen and Egger (1914) employed definite chemical mediums to determine the relative nutritional value of glycocoll, alanin, and asparagin for *B. prodigiosus*. Nawiasky (1908) studied the ability of *Proteus vulgaris* to attack various amino-acids, asparagin, creatin, taurin, uric acid, and urea. Frouin and Ledebt (1912) cultivated *B. pyocyaneus* on a medium in which asparagin constituted the only source of nitrogen.

The use of "synthetic" mediums for the purpose of isolating from the intestine certain bacteria which attack the products of digestion has been suggested by several workers. Among these should be mentioned Berthelot (1911, 1912, and 1917) who has recommended several suitable mediums and has studied a number of the isolated organisms. Mellanby and Twort (1912) isolated a bacillus of the colon group which possessed the power of converting histidin, by decarboxylation, into ergamin. Jones (1918) records the isolation of an organism called *Bacillus aminophilus* which was capable of producing this same product from histidin. A similar organism had been previously described by Berthelot and Bertrand (1912).

In the foregoing publications attention has been centered, for the most part, on a single species of bacterium. In contrast to the many investigations of such nature which occur in the literature, one finds that comparatively few attempts have been made to determine the relative values of the various simple and chemically-definite nitrogenous foodstuffs for different species. This field of investigation would seem to be an important one, for by obtaining a knowledge of the availability of these substances we might hope to construct, eventually, a medium of known chemical structure on which even the more exacting pathogens would develop. The advantages of such a medium for the study of metabolism, toxin production, the structure of toxins, etc., are obvious. At the present time, however, we are far from realizing this ideal.

As a step in this direction Lepière (1903) investigated the availability of certain glucoproteins. The glucoproteins were added in quantities of 1.5-3 gm. to a medium composed of:

Water.....	100 c.c.
NaCl.....	0.5 gm.
MgSO ₄	0.05 gm.
Calcium glycerophosphate.....	0.2-0.3 gm.
KHCO ₃	0.1-0.2 gm.

Only the simpler glucoproteins containing 6-11 carbon atoms were employed. In some cases glucose, saccharose, or glycerol, was added as an additional source of carbon. Of 45 different micro-organisms tested by Lepière, 14 pathogenic and 23 saprophytic species were able to develop on all the glucoproteins, while *B. diphtheriae*, *B. anthracis*, *B. tetani*, a species of streptococcus, the vibron septique and *B. tuberculosis* required glucoproteins containing 8-11 carbon atoms. In the light of more recent knowledge, however, it would seem that Lepière's glucoproteins were not as definite structurally as he supposed.

Sullivan (1906), employed many different "synthetic" mediums in his study on bacterial pigments, but the number of nitrogenous compounds used was somewhat limited. After testing the ability of various pigment producers to develop on his mediums he recommends one composed of asparagin, 1%; magnesium sulphate, 0.02%; di-potassium hydrogen phosphate, 0.1%; ammonium lactate, 0.05%; sodium chlorid, 0.5%; potassium nitrate, 0.02%; glycerol, 1%, and agar, 2%.

Probably the most noteworthy study of the availability of nitrogenous compounds of definite chemical structure is that of Galimard and Lacomme (1907). These authors employed as a substratum the inorganic salts used by Lepière plus 1.5% of glycerol. The nitrogen was supplied by various amino-acids and urea, added either singly or in combinations. In this way glycocoll, leucin, aspartic acid, tyrosin, phenylalanin, lysin, arginin, and urea were subjected to the action of 24 different species of bacteria. Arginin was found to be superior as a single source of nitrogen, since 13 of the 24 species were able to develop when this amino-acid was present. Phenylalanin failed entirely to support growth, while lysin and aspartic acid each supported the growth of but a single species—*B. pyocyaneus*. Urea supported the growth of but 2 species while glycocoll, leucin, and tyrosin gave somewhat better results.

In addition, 11 different combinations of the above amino-acids were tried. One medium composed of glycocoll, 0.3%; leucin, 0.05%; tyrosin, 0.01%; aspartic acid, 0.1%, and arginin chlorhydrate, 0.23%, and a second of glycocoll, 0.9%, and arginin chlorhydrate, 0.1% proved to be the 2 most satisfactory combinations. It is noteworthy that *B. diphtheriae*, *B. tetani*, *B. tuberculosis*, *B. typhosus*, and several species of cocci failed to develop in every case.

Quite recently Gordon (1917) attempted to ascertain whether the commoner pathogenic bacteria exhibit any marked difference in regard to their individual capacity to break down certain nitrogenous substances of known chemical composition.

He employed a medium consisting of:

K ₂ HPO ₄	0.1%
MgSO ₄	0.2
CaCl ₂	0.01
NaCl	0.5%
Washed agar	3%
Various ammonium salts, amides, or amino-acids.....	1%
(unless only sparingly soluble)	

This medium was tried both with and without the addition of glucose. It was found that where glucose was present as a source of carbon, bacteria, such as *B. coli*, *B. paratyphosus*, *B. pyocyaneus*, and *B. proteus* can satisfy their nitrogen requirement with either ammonium salts, sundry amides, amino-acids (glycocoll, alanin, aspartic acid) or asparagin. On the other hand, *B. typhosus*, Friedländer's bacillus, *B. diphtheriae*, *B. pseudo-diphtheriae*, and several species of staphylococci and streptococci appear to possess no such ability. The cholera vibrio and *B. dysenteriae* (Flexner) were found to be capable of utilizing certain of the amino-acids, but not the ammonium salts.

Summing up, we see that attention is being more and more centered on the simple nitrogenous bodies instead of those of complex nature, and that promising results have been secured by employing a heterogeneous mixture of the products of proteolytic cleavage. Hence, the present need would appear to be a thorough study of the isolated and purified products of protein hydrolysis.

The aim of the present investigation has been to gain some knowledge of the ability of various species of bacteria to utilize these simpler nitrogenous substances of known chemical structure. Chief emphasis has been placed on the amino-acids, the purins and extractives, and various combinations of these substances.

EXPERIMENTAL

The following combination was adopted as the basic medium:

Distilled ammonia-free water.....	1,000	c.c.
NaCl	5.0	g.
MgSO ₄	0.2	g.
CaCl ₂	0.1	g.
KH ₂ PO ₄	1.0	g.
K ₂ HPO ₄	1.0	g.
Glycerol	30.0	g.

To this constant substratum the nitrogenous bodies to be tested were added, usually in quantities sufficient to make 0.1%.

This constant substratum is essentially a modification of Uschinsky's medium from which the nitrogen-containing compounds—asparagin and ammonium lac-

tate—have been withdrawn and the amount of glycerol reduced to 3%. The primary potassium phosphate was added to neutralize the alkaline reaction imparted by the di-potassium phosphate. Several determinations of the hydrogen ion concentration, made by the colorimetric method, showed that the medium described in the foregoing had a P_H value of 6.6.

Glycerol was used as a source of energy, as its presence was found to favor the growth of the various organisms. Dextrose—1%—was also tried and, with a few minor exceptions, gave essentially the same results as were secured with glycerol. On the whole, there appeared to be somewhat less acid formed from the glycerol, and as this allowed greater freedom of development it was given preference over the dextrose.

To this constant substratum were added the various simple nitrogenous compounds, either singly or in combinations. The resultant medium is clear and colorless and does not require filtration.* It was filled into ordinary test tubes and sterilized in the autoclave at 10 or 12 lbs. extra pressure for 15 minutes. Liquid mediums only were employed.

Only those nitrogenous compounds which were of undoubted purity were used. The writers are indebted to Prof. F. P. Underhill for the following: valin, glutamic acid, phenylalanin, tyrosin, lysin picrate, urea, uric acid, hypoxanthin, allantoin, and creatin. Glycocoll, asparatic acid, and asparagin, were procured from the Eimer and Amend Co. The tryptophan, obtained from the same company, was labeled Grübler and Co., Leipzig. To prepare leucin, the writers hydrolyzed serum globulin (horse) with sulphuric acid. The leucin fraction was separated and the leucin purified after the method of Ehrlich and Wendel (1908). Histidin monochlorid was prepared from sheep blood according to Knoop (1907). Lysin dichlorid was obtained from lysin picrate after the method of Kossel (1898). Taurin was prepared from desiccated ox-bile (Hammarsten and Hedin, 1914). In every case the products thus secured were subjected to repeated crystallization to insure their purity.

All inoculations, unless otherwise indicated, were made with the tip of a bent platinum rod from 24-hour slant agar cultures of the micro-organisms in question, care being taken to introduce as small a quantity as possible of the culture.

Several experiments in which inoculations were made from old agar cultures showed that the results obtained were variable and unreliable, due presumably to the introduction of enzymes, dead bacterial cells, or other nitrogenous matter into the synthetic medium.

Precautions were taken to prevent absorption of atmospheric ammonia during the period of incubation. As free ammonia may be given off from cultures in the ordinary peptone meat mediums during the course of bacterial development, all tubes of the various "synthetic" mediums, when placed in an incubator containing other cultures, were enclosed in small metal boxes to prevent, in so far as possible, the absorption of gaseous ammonia.

The McFarland nephelometer was employed in estimating luxuriance of growth. Standards were made to give the following densities: 0.25, 0.5, 0.75, 1.0, 1.5, 2.0, 3.0, 4.0, 5.0, and 7.0. It was found unnecessary to go above the last figure. These terms represent the actual number of cc of the barium chlorid solution added to the sulphuric acid. Five cc amounts of the standard suspensions were filled into test tubes of a diameter equal to that of the tubes used to contain the culture mediums. All standard tubes were then sealed in a flame to prevent evaporation.

* The turbidity which is quite apparent while the medium is hot completely disappears on cooling.

To estimate the amount of growth the culture in question was compared with those tubes of the standard series most nearly approximating it. The comparison was made against a black background in strong indirect light. Although this method, as used in the present investigation, does not give an accurate estimate of the number of bacterial cells in a given culture, it nevertheless gives a series of gradients which express fairly accurately the degree of turbidity resulting from the growth of the micro-organisms in question. It possesses the advantage of furnishing definite chemical standards which do not deteriorate and by means of which the results obtained with varying nitrogenous compounds and by different investigators may be more readily correlated. In the case of a flocculent, stringy, or "mucilaginous" growth, comparison is made with difficulty or cannot be made at all. This was found to be the case with cultures of *B. cereus*, *B. subtilis*, and *B. mycoides* and also to a certain extent with the older cultures of *B. pyocyaneus*, *B. cloacae*, *B. acidi* (*lactici*) and occasionally others. Nevertheless, in the writers' opinion, this method has proved to be on the whole quite reliable and much to be preferred to the expression of growth by the terms, slight, good, etc.

To gain an idea of the rapidity of development, readings were taken by comparison with the standards at intervals of 24 hours, 48 hours, 4 days and 7 days, after inoculation. In a number of cases the period of observation was extended to 2 weeks, but as little additional information was gained by this procedure it was discontinued.

To check the first series of results in any given medium, transplants were made from these tubes into a second series of the same medium. This inoculation was made within the first 24 hours—wherever sufficient growth had been attained to permit this—by means of a small platinum loop about 1 mm. in diameter. To further check the results stains were made and agar slants stroked from the cultures showing no growth or in those cases where the results were questionable.

MONO-AMINO ACIDS AS THE ONLY SOURCE OF NITROGEN

The following mono-amino acids were employed: glycocoll, valin, leucin, aspartic acid, and glutamic acid. All were used in 0.1% concentration. In addition, the glycocoll and aspartic acid were made up into 0.3% solutions and submitted to the same culture tests. The aspartic and glutamic acid mediums were rendered neutral to litmus by the addition of sodium hydroxid solution. Since the results obtained with the different mono-amino acids are essentially the same, only 1 of the 7 complete tables is presented here (see Table 1).

B. subtilis, *B. cereus* and *B. mycoides* produced flocculent growths, so it was impossible to determine their luxuriance by the barium sulphate standards. The extent of growth is expressed by + or ++ signs, the former indicating slight and the other fair growth.

Several of the organisms employed are conspicuous because of their inability to develop on any of the mediums containing the above amino-acids. They are *B. anthracis*, *Proteus zenkeri*, *B. typhosus*, *B. dysenteriae*, *B. abortus*, *B. pul-lorum*, *B. diphtheriae*, *B. hoffmanni* and all of the cocci studied. Of the pathogens employed only *B. pyocyaneus*, and paratyphoid bacilli, A and B, *B. sanguinarium*, and the cholera vibrio were able to utilize the amino-acids as a source of nitrogen. The common saprophytic bacilli exhibited for the most

Note.—For additional tables and other data not included in this paper the reader is referred to the doctorate thesis (S. A. Koser) in the Yale University Library.

part an abundant growth, although the spore-formers, *B. subtilis*, *B. cereus* and *B. mycoides* developed rather slowly, scantily and irregularly.

The inability of leucin to function as an important source of nitrogen for bacteria is of particular interest, in view of the recent publication of Zunz and György (1916). As a result of their own investigation these authors claim that leucin promotes luxuriant development of *B. coli*, and that *B. typhosus*

TABLE 1
CONSTANT SUBSTRATUM PLUS 0.1% GLYCOCOLL

	Degree of Growth at Expiration of			
	24 Hrs.	48 Hrs.	4 Days	7 Days
<i>B. cereus</i> (Frankland).....	0.0	+?	+	+
<i>B. subtilis</i> (Cohn).....	0.0	?	+	+
<i>B. mycoides</i> (Flügge).....	0.0	?	+	+
<i>B. anthracis</i>	0.0	0.0	0.0	0.0
<i>B. fluorescens</i> liq.	0.25	1.5	3.0	4.0
<i>B. fluorescens</i> nonliq.	0.5	1.0	1.5	2.0
<i>B. pyocyaneus</i>	0.5	1.0	2.0	2.0
<i>B. prodigiosus</i>	0.5	1.0	1.5	2.0
<i>B. ruber balticus</i>	0.0	0.25	1.0	1.5
<i>Proteus vulgaris</i>	0.0	0.25	0.25	0.25*
<i>Proteus mirabilis</i>	?	0.25	0.25	0.25
<i>Proteus zenkeri</i>	0.0	0.0	0.0	0.0
<i>B. capsulatus</i>	0.5	1.0	1.0	1.0
<i>B. coli</i> (104).....	0.75	1.0	1.5	1.5
<i>B. coli</i> (U).....	0.5	1.0	1.5	2.0
<i>B. aerogenes</i>	0.5	4.0	5.0
<i>B. cloacae</i>	0.5	1.0	1.5	3.0
<i>B. acidi lactici</i>	0.5	1.0	1.5	2.0
<i>B. paratyphosus</i> A.....	0.5	0.75	0.75	1.0
<i>B. paratyphosus</i> B.....	0.25	0.75	1.0	1.5
<i>B. pullorum</i>	0.0	0.0	0.0	0.0
<i>B. sanguinarium</i>	0.0	0.0	0.25	0.25
<i>B. typhosus</i>	0.0	0.0	0.0	0.0
<i>B. dysenteriae</i> (Shiga).....	0.0	0.0	0.0	0.0
<i>B. abortus</i> (Bang).....	0.0	0.0	0.0	0.0
<i>B. abortus</i> (Wis.).....	0.0	0.0	0.0	0.0
<i>B. hoffmanni</i>	0.0	0.0	0.0	0.0
<i>B. diphtheriae</i> (Park No. 8).....	0.0	0.0	0.0	0.0
<i>B. diphtheria</i> (Y. M. S.).....	0.0	0.0	0.0	0.0
<i>V. cholerae</i> (R).....	0.0	?	1.0	2.0
<i>Sarcina lutea</i>	0.0	0.0	0.0	0.0
<i>Sarcina aurantiaca</i>	0.0	0.0	0.0	0.0
<i>Mic. tetragenus</i>	0.0	0.0	0.0	0.0
<i>Mic. cereus flavus</i>	0.0	0.0	0.0	0.0
<i>Staph. aureus</i>	0.0	0.0	0.0	0.0
<i>Staph. albus</i>	0.0	0.0	0.0	0.0
<i>Str. pyogenes</i> (309).....	0.0	0.0	0.0	0.0
<i>Str. pyogenes</i> (346).....	0.0	0.0	0.0	0.0
<i>Streptothrix</i>	0.0	0.0	+	+

* Failed to grow on reinoculation into 2nd tube.

finds sufficiently favorable conditions for slight but unmistakable growth. In our experience leucin has been in no wise different from glycocoll or any of the other mono-amino acids employed.

Uschinsky's medium was employed for the purpose of comparison. The results with all of the test organisms were so nearly like those furnished by the above experimental mediums that they are not presented here in tabulated form.

A full set of control tests was made also with the constant substratum. All of the organisms failed to develop except in a very few instances in which on prolonged incubation a very light or questionable growth occurred, in all probability as the result of the absorption of minute quantities of ammonia from the atmosphere. The few organisms which seemed to possess this ability were later found to appropriate to their own needs the ammonia nitrogen of ammonium phosphate.

In a medium containing free aspartic or glutamic acid as the only source of nitrogen the results are very uncertain, as is shown also in the conflicting data which have been reported from other laboratories recently. When such a medium is neutralized the aspartic or glutamic acid will play essentially the same rôle as glycocoll, leucin, etc.

TABLE 2

SHOWING THE EFFECT OF DECREASING THE SUPPLY OF NITROGEN
Aspartic Acid (neutralized) Added to the Usual Constant Substratum in Varying Amounts.
Record of Growth Made at Expiration of 7 Days

	None (Control)	0.1%	0.02%	0.002%	0.001%	0.0001%
<i>B. cereus</i>	++	+	+	?	0.0
<i>B. subtilis</i>	+	+	?	0.0	0.0
<i>B. mycoides</i>	+	+	?	?	0.0 ?
<i>B. fluorescens</i>	0.0	1.5	1.5	0.75	0.25	0.0
<i>B. fluorescens nonliq.</i>	3.0	2.0	0.5	0.25	?
<i>B. pyocyaneus</i>	0.0 ?	1.5	1.5	0.75	0.25	?
<i>B. prodigiosus</i>	4.0	1.5	0.5	?	0.0
<i>Proteus mirabilis</i>	0.5	0.25	0.25	0.0	0.0
<i>B. capsulatus</i>	0.0	5.0	1.5	0.75	?	0.0
<i>B. coli</i> (104).....	0.0	3.0	1.5	0.5	0.0	0.0
<i>B. coli</i> (U).....	4.0	1.5	0.5	?	0.0
<i>B. cloacae</i>	0.0	4.0	1.5	0.5	0.25	0.0 ?
<i>B. acid</i>	0.0	5.0	1.5	0.5	?	0.0
<i>B. paratyphosus A</i>	1.5	1.5	0.5	?	0.0
<i>B. paratyphosus B</i>	1.5	1.5	0.5	?	0.0
<i>B. sanguinarium</i>	1.0	0.5	0.25	?	0.0
<i>V. cholerae</i> (R).....	1.5	0.5	0.5	0.0	0.0
<i>V. cholerae</i> (Y. M. S.).....	0.0	1.0	1.0	0.5	0.0	0.0

A concentration of 0.1% of the mono-amino acids supplies sufficient nitrogen for the organisms which can utilize it at all, since the results were practically the same whether the amino-acids constituted 0.1 or 0.3% of the medium. In order to obtain further information as to the minimum amount of amino-acid required to visibly affect the luxuriance of bacterial growth, aspartic acid was employed in diminishing quantities, namely, 0.1%, 0.02%, 0.002%, 0.001%, 0.0001% (Table 2). As the amount of amino-acid nitrogen was decreased bacterial multiplication was lessened until at a concentration of 0.0001% there was little if any development. Certain organisms which in 0.1% aspartic acid attain a luxuriance of growth of 3.0, 4.0 or 5.0, are unable to develop beyond 1.5 on the barium sulphate scale when the aspartic acid is decreased to 0.02%. All of these tests were made with neutralized aspartic acid medium, using, as at all other times, the substratum described earlier in this paper.

VIABILITY EXPERIMENTS IN AMINO-ACID MEDIUMS

The following plan was adopted for determining the ability of bacteria to preserve their normal growth characteristics during long periods of cultivation

in the amino-acid mediums. Ten representative organisms were selected and carried through 12 successive cultures in 0.1% aspartic acid medium rendered neutral to litmus. The inoculations from tube to tube were made with a 1 mm. loop, and at intervals of 24 hours, or, in the case of the slowly-growing forms, as soon thereafter as sufficient growth was obtained.

In Table 3 are given figures for the first, eighth, and the twelfth or last of the consecutive cultures. As no diminution in the luxuriance of growth had occurred up to this time further cultures were not made. The last set of cultures was kept at room temperature for a period of 3 months. Slants of nutrient agar were streaked from these tubes every 2 weeks, with the following results.

TABLE 3
SHOWING THE RESULT OF CONSECUTIVE TRANSPLANTS IN AMINO-ACID MEDIUM:
0.1% Aspartic Acid—Neutralized

	24 Hrs.	48 Hrs.	4 Days	7 Days
1st Culture:				
<i>B. cereus</i>	0.0	+?	++	++
<i>B. fluorescens</i>	0.5	1.5	3.0	4.0
<i>B. pyocyaneus</i>	0.5	1.0	1.5	1.5
<i>B. prodigiosus</i>	0.25	1.5	2.0	3.0
<i>Proteus vulgaris</i>	0.0	0.0	0.0 ?	0.0 ? *
<i>B. coli</i> (U).....	0.5	1.5	3.0	4.0
<i>B. paratyphosus</i> B.....	0.5	1.0	1.0	1.0
<i>B. cloacae</i>	0.5	2.0	3.0	3.0
<i>B. acidi</i>	1.0	2.0	3.0	4.0
<i>V. cholerae</i> (R).....	0.0	?	0.5	1.0
8th Consecutive Culture:				
<i>B. cereus</i>	?	+	+
<i>B. fluorescens</i>	1.0	2.0	4.0
<i>B. pyocyaneus</i>	0.5	1.5	1.5	1.5
<i>B. prodigiosus</i>	0.5	2.0	2.0	3.0
<i>Proteus vulgaris</i>	0.0	0.0	0.0	0.0 *
<i>B. coli</i> (U).....	2.0	3.0	4.0	5.0
<i>B. paratyphosus</i> B.....	0.5	1.0	1.0	1.0
<i>B. cloacae</i>	1.0	2.0	3.0	3.0
<i>B. acidi</i>	1.0	1.5	4.0	5.0
<i>V. cholerae</i> (R).....	0.25	1.0	1.0
12th Consecutive Culture:				
<i>B. cereus</i>	+	++	++
<i>B. fluorescens</i>	0.5	1.5	3.0	5.0
<i>B. pyocyaneus</i>	1.0	2.0	2.0	2.0
<i>B. prodigiosus</i>	1.0	1.5	2.0	2.0
<i>Proteus vulgaris</i>	0.0	0.0	0.0	0.0 *
<i>B. coli</i> (U).....	1.5	4.0	4.0	5.0
<i>B. paratyphosus</i> B.....	0.5	1.0	1.5	1.0
<i>B. cloacae</i>	1.0	2.0	3.0	3.0
<i>B. acidi</i>	1.5	3.0	4.0	5.0
<i>V. cholerae</i> (R).....	0.5	1.5	1.5

* Good growth was obtained when agar slants were stroked from the cultures at 7th day.

B. paratyphosus B and the cholera vibrio were found to have died by the end of the 2nd week, while *B. coli* and *B. acidi lactici* failed to grow when transplanted to the agar after a month. These 4 organisms produced sufficient acid, however, from the glycerin in the constant substrate to give a positive reaction with methyl red. The remaining amino-acid cultures, which gave only a pure yellow or neutral tint with methyl red, remained viable to the termination of the experiment, as did control cultures of the same organisms in bouillon.

Repeated transplants in the amino-acid medium did not result in any apparent loss of viability. Indeed, in a few instances, growth became more luxuriant after each successive transfer.

THE RELATIVE SIGNIFICANCE OF LARGE AND SMALL AMOUNTS OF INOCULUM
ON RAPIDITY AND LUXURIANCE OF GROWTH

In the ordinary transfer of bacteria from one medium to another by means of the platinum straight wire or loop, hundreds of thousands, and at times millions of living bacterial cells are carried over, in spite of all possible care. After some preliminary experiments it was found that by inoculating tubes of sterile water and making certain dilutions, it was possible to transfer, with the aid of a 4 mm. loop, fairly definite numbers, 200-600 cells.

TABLE 4
CONSTANT SUBSTRATUM PLUS 0.1% LYSIN
Lysin Dichlorid 0.15%—Neutralized with Na_2CO_3

	Degree of Growth at Expiration of			
	24 Hrs.	48 Hrs.	4 Days	7 Days
<i>B. cereus</i>	0.0	+	+	+
<i>B. subtilis</i>	0.0	0.0	0.0	0.0
<i>B. ramosus</i>	+	+	+	+
<i>B. anthracis</i>	0.0	0.0	0.0	0.0
<i>B. fluorescens</i>	0.5	1.0	2.0	3.0
<i>B. fluorescens nonliq.</i>	0.5	1.0	3.0	4.0
<i>B. pyocyaneus</i>	1.0	1.0	1.5	2.0
<i>B. prodigiosus</i>	?	0.5	1.0	1.5
<i>B. ruber</i>	0.0	0.0	0.5	1.5
<i>Proteus vulgaris</i>	0.0	?	0.25	0.25
<i>Proteus mirabilis</i>	0.25	0.5	0.5	0.5 *
<i>Proteus zenkeri</i>	0.0	0.0	0.0	0.0
<i>B. capsulatus</i>	0.5	1.0	1.5	2.0
<i>B. coli</i> (104).....	0.5	0.75	1.0	1.5
<i>B. coli</i> (U).....	0.5	1.0	1.0	2.0
<i>B. aerogenes</i>	0.25	0.5	1.0	2.0
<i>B. cloacae</i>	1.0	1.0	1.5	2.0
<i>B. acidi lactici</i>	1.0	1.0	1.0	1.5
<i>B. paratyphosus A</i>	0.5	0.5	1.0	1.0
<i>B. paratyphosus B</i>	?	0.5	0.5	1.0
<i>B. pullorum</i>	0.0	0.0	0.0	0.0
<i>B. sanguinarium</i>	0.0	0.0	0.0	0.0
<i>B. typhosus</i>	0.0	0.0	0.0	0.0
<i>B. dysenteriae</i> (Shiga).....	0.0	0.0	0.25	?
<i>B. abortus</i> (Bang).....	0.0	0.0	0.0	0.0
<i>B. abortus</i> (Wis.).....	0.0	0.0	0.0	0.0
<i>B. hoffmanni</i>	0.0	0.0	0.0	0.0
<i>B. diphtheriae</i> (Park No. 8).....	0.0	0.0	0.0	0.0
<i>B. diphtheriae</i> (Y. M. S.).....	0.0	0.0	0.0	0.0
<i>V. cholerae</i> (R).....	0.5	1.0	1.0	1.0
<i>V. cholerae</i> (Y. M. S.).....	0.0	0.0	0.25	1.0
<i>Sarcina lutea</i>	0.0	0.0	0.0	0.0
<i>Sarcina aurantiaca</i>	0.0	0.0	0.0	0.0
<i>Mic. tetragenus</i>	0.0	0.0	0.0	0.0
<i>Mic. flavus</i>	0.0	0.0	0.0	0.0
<i>Staph. aureus</i>	0.0	0.0	0.0	0.0
<i>Staph. albus</i>	0.0	0.0	0.0	0.0
<i>Str. pyogenes</i> 209.....	0.0	0.0	0.0	0.0
<i>Str. pyogenes</i> 346.....	0.0	0.0	0.0	0.0
<i>Streptothrix</i>	0.0	0.0	+	++

* Failed to develop when reinoculated into 2nd tube.

Two different amino-acid mediums, the one containing valin, and the other aspartic acid as the sole source of nitrogen, were each inoculated, by the above dilution method, with the different test organisms. As large inoculums are accompanied by appreciatble amounts of enzymes, in case they are elaborated, as has been shown in this laboratory, the smallest amounts that were practicable were employed. These experiments were repeated several times.

TABLE 5
CONSTANT SUBSTRATUM PLUS 0.1% PHENYLALANIN

	Degree of Growth at Expiration of			
	24 Hrs.	48 Hrs.	4 Days	7 Days
<i>B. cereus</i>	?	+	++	++
<i>B. subtilis</i>	0.0	0.0	+	+
<i>B. ramosus</i>	0.0	0.0	++	++
<i>B. anthracis</i>	0.0	0.0	0.0	0.0
<i>B. fluorescens</i>	0.5	1.0	1.0	1.5
<i>B. fluorescens nonliq.</i>	0.25	0.5	0.5	1.0
<i>B. pyocyaneus</i>	0.25	0.5	1.0	1.0
<i>B. prodigiosus</i>	0.0	?	0.5	1.0
<i>B. ruber</i>	0.0	?	0.5	1.5
<i>Proteus vulgaris</i>	0.0	0.0	0.0	?
<i>Proteus mirabilis</i>	0.0	?	0.25	0.25*
<i>Proteus zenkeri</i>	0.0	0.0	0.0	0.0
<i>B. capsulatus</i>	0.5	1.0	1.0	1.0
<i>B. coli</i> (104).....	0.25	0.5	0.5	1.0
<i>B. coli</i> (U).....	?	0.25	0.5	0.5
<i>B. cloacae</i>	0.25	0.5	1.0	1.0
<i>B. acidi</i> (lactici).....	0.25	0.25	1.0	1.0
<i>B. paratyphosus A</i>	0.25	0.5	0.5	0.5
<i>B. paratyphosus B</i>	?	?	0.5	0.5
<i>B. pullorum</i>	0.0	0.0	0.0	0.0
<i>B. sanguinarium</i>	0.0	?	?	?
<i>B. typhosus</i>	0.0	0.0	0.0	0.0
<i>B. dysenteriae</i> (Shiga).....	0.0	0.0	0.0	0.0
<i>B. abortus</i> (Bang).....	0.0	0.0	0.0	0.0
<i>B. abortus</i> (Wis.).....				
<i>B. hoffmanni</i>	0.0	0.0	0.0	0.0
<i>B. diphtheria</i> (Park No. 8).....	0.0	0.0	0.0	0.0
<i>B. diphtheria</i> (Y. M. S.).....				
<i>V. cholerae</i> (R).....	0.0	0.0	0.0	0.5
<i>V. cholerae</i> (Y. M. S.).....				
<i>Sarcina lutea</i>	0.0	0.0	0.0	0.0
<i>Sarcina aurantiaca</i>	0.0	0.0	0.0	0.0
<i>Mic. tetragenus</i>	0.0	0.0	0.0	0.0
<i>Mic. cereus flavus</i>	0.0	0.0	0.0	0.0
<i>Staph. aureus</i>	0.0	0.0	0.0	0.0
<i>Staph. albus</i>	0.0	0.0	0.0	0.0
<i>Str. pyogenes</i> 346.....	0.0	0.0	0.0	0.0
<i>Streptothrix</i>	0.0	0.0	?	?

* On subculture to 2nd tube, growth was so slight as to be questionable.

By comparison with the results shown in Table 1 and similar tables not included in this paper, it was readily seen that during the first 24-48 hours the growth resulting from the small inoculum is not as luxuriant as that following the usual method. The relatively few cells which are carried over are able, however, to develop, and at the end of several days produce as luxuriant growth as is obtained with the larger inoculums, with the exception of *Proteus vulgaris*, which failed to grow, and of the *subtilis* group, which multiplied very

slowly or not at all. The other organisms seem to be able to assimilate the amino-acid nitrogen without the aid of enzyme action, particularly *B. capsulatus*, *B. coli*, *B. cloacae* and *B. acidilactici*.

TABLE 6
CONSTANT SUBSTRATUM PLUS 0.1% HISTIDIN
Used as Histidin Monochlorid 0.135% and Neutralized

	Luxuriance of Growth at Expiration of			
	24 Hrs.	48 Hrs.	4 Days	7 Days
<i>B. cereus</i>	0.0	+	++	++
<i>B. subtilis</i>	0.0	0.0	?
<i>B. ramosus</i>	0.0	0.0	+
<i>B. anthracis</i>	0.0	0.0	0.0	0.0
<i>B. fluorescens</i>	0.5	1.5	3.0	4.0
<i>B. fluorescens nonliq.</i>	0.5	1.0	3.0	5.0
<i>B. pyocyaneus</i>	1.0	1.5	1.5	2.0
<i>B. prodigiosus</i>	0.5	1.5	2.0	2.0
<i>B. ruber</i>	0.0	0.25	1.5	2.0
<i>Proteus vulgaris</i>	0.0	?	?	0.25*†
<i>Proteus mirabilis</i>	0.0	0.0	0.0	0.0 *
<i>Proteus zenkeri</i>	0.0	0.0	0.0	0.0
<i>B. capsulatus</i>	1.0	1.5	1.5	2.0
<i>B. coli</i> (104).....	0.25	0.25	0.5	0.5
<i>B. coli</i> (U).....	0.25	0.25	0.5	0.5 †
<i>B. aerogenes</i>	0.75	2.0	2.0
<i>B. cloacae</i>	0.5	1.5	2.0	3.0
<i>B. acidilactici</i>	0.0	?	0.25	0.5
<i>B. paratyphosus A</i>	0.5	0.75	1.0	1.5
<i>B. paratyphosus B</i>	0.5	0.5	1.0	1.5
<i>B. pullorum</i>	0.0	0.0	0.0	0.0
<i>B. sanuinarium</i>	0.25	0.25	0.25	0.25
<i>B. typhosus</i>	0.0	0.0	0.0	0.0
<i>B. dysenteriae</i> (Shiga).....	0.0	0.0	0.0	0.0
<i>B. abortus</i> (Bang).....	0.0	0.0	0.0	0.0
<i>B. abortus</i> (Wis.).....	0.0	0.0	0.0	0.0
<i>B. hoffmanni</i>	0.0	0.0	0.0	0.0
<i>B. diphtheriae</i> (Park No. 8).....	0.0	0.0	0.0	0.0
<i>B. diphtheriae</i> (Y. M. S.).....	0.0	0.0	0.0	0.0
<i>V. cholerae</i> (R).....	0.25	0.5	1.0	1.5
<i>V. cholerae</i> (Y. M. S.).....	0.0	0.0	0.0	0.25
<i>Sarcina lutea</i>	0.0	0.0	0.0	0.0 ?
<i>Sarcina aurantiaca</i>	0.0	0.0	0.0	0.0
<i>Mic. tetragenus</i>	0.0	0.0	0.0	0.0
<i>Mic. flavus</i>	0.0	0.0	0.0	0.0
<i>Staph. aureus</i>	0.0	0.0	0.0	0.0
<i>Staph. albus</i>	0.0	0.0	0.0	0.0
<i>Staph. citreus</i>	0.0	0.0	0.0	0.0
<i>Strep. pyogenes</i> (U).....	0.0	0.0	0.0	0.0
<i>Cladotrix</i>	0.0	?	+

* Yellow.

† Failed to develop when re inoculated into 2nd tube.

DIAMINO, AROMATIC, AND HETEROCYCLIC AMINO-ACIDS AS SOURCES OF NITROGEN

The substances comprising this mixed group were lysin, tyrosin, phenylalanin, histidin, and tryptophan.

Since lysin possesses 2 amino groupings in the molecule, a study of its availability as a source of nitrogen appeared promising. In Table 4 are given

the results obtained with a medium containing lysin and the substrate employed throughout the investigation. Contrary to expectations, lysin apparently has no advantage over the mono-amino acids, at least in so far as its deportment toward the more exacting pathogenic species of bacteria is concerned. *B. dysenteriae* grew very slightly in one instance, but in subsequent tests the results were negative.

The availability of tyrosin and phenylalanin is represented in Table 5. The response to the different organisms employed was somewhat less than that secured with the mono-amino-acid mediums (See Table 1). A marked difference is noticeable in the luxuriance of the growth. For instance, with both strains of the colon bacillus and the paratyphoid organisms a less dense growth is obtained by the use of these aromatic amino-acids than with the mono-amino. The same is true of the chromogenic forms. It should be stated here that the quantity of tyrosin in solution in the tyrosin medium (0.03%) was limited by its slight solubility, and may in a measure at least be responsible for the lighter growth in this medium. Both tyrosin and phenylalanin are distinctly inferior to the other amino-acids employed in this work.

The heterocyclic amino-acid, histidin, is of no greater value as a source of available nitrogen than the mono-amino acids and lysin (Table 6). A peculiar coloration of this medium was brought about by *Proteus vulgaris* and *P. mirabilis*. During several days' incubation the medium assumed a light color which by the end of a week became a rich yellow. A similar phenomenon was observed when the histidin was replaced by tryptophan. Cornish and Williams (1917) had called attention to light pinkish brown or amber color production by the proteus group in a histidin or tryptophan medium.

Tryptophan appears to possess a slight advantage over the nitrogenous substances thus far considered. In addition to the organisms which usually develop in the amino-acid mediums, others produced a visible clouding. In some instances, however, ability to grow was lost on further transplantation in the same medium. This was particularly true of *B. anthracis*, *B. dysenteriae*, and *Sarcina aurantiaca*. On the other hand, *B. typhosus* was capable of producing light growth through the several successive transfers.

Proteus vulgaris and *P. mirabilis* brought about a distinct yellow to amber color in the tryptophan medium after several days' incubation. A very light color was apparent also in both cultures of the cholera vibrio in the same medium. This observation indicates a similarity in the nature of the decomposition of tryptophan and histidin, at least in so far as the unusual color product is concerned.

OTHER SOURCES OF NITROGEN

In addition to the various substances employed in the preceding experiments a number of other chemically-definite sources of nitrogen were submitted to the same tests, namely, ammonium phosphate, urea, taurin, creatin, hypoxanthin, uric acid and allantoin.

Diammonium hydrogen phosphate yielded results but slightly different from those obtained with the amino-acids. Even such organisms as the paratyphoid bacilli and *B. sanguinarium* are able to satisfy their nitrogen requirements in the presence of this ammonium salt. However, both strains of the cholera vibrio refused to grow, although the experiment was repeated several times. This observation is in accordance with that of Gordon (1917) who states that the cholera vibrio is capable of utilizing certain amino-acids, but not ammonium salts.

Urea is somewhat inferior to the amino-acids as nitrogenous food for bacteria, while taurin and creatin failed to supply available nitrogen in almost every case.

TABLE 7

URIC ACID, 0.1%, ADDED TO CONSTANT SUBSTRATUM, FROM WHICH THE 0.1% KH_2PO_4 WAS OMITTED

	Degree of Growth at Expiration of			
	24 Hrs.	48 Hrs.	4 Days	7 Days
<i>B. cereus</i>	0.0	0.0	0.0	0.0
<i>B. subtilis</i>	0.0	0.0	0.0	0.0
<i>B. ramosus</i>	0.0	0.0	0.0	0.0
<i>B. anthracis</i>	0.0	0.0	0.0	0.0
<i>B. fluorescens</i>	0.5	1.5	3.0	4.0
<i>B. fluorescens nonliq.</i>	0.25	0.5	2.0	3.0
<i>B. pyocyaneus</i>	0.25	1.0	1.5	1.5
<i>B. prodigiosus</i>	0.5	1.0	1.5	1.5
<i>B. ruber</i>	0.0	0.0	0.5	1.0
<i>Proteus vulgaris</i>	0.0	0.0	0.0	0.0
<i>Proteus mirabilis</i>	0.0	0.0	0.0	0.0
<i>Proteus zenkeri</i>	0.0	0.0	0.0	0.0
<i>B. capsulatus</i>	0.5	1.0	2.0	2.0
<i>B. coli</i> (104).....	0.0	0.0	0.0	0.0 ?
<i>B. coli</i> (U).....	0.0	0.0	0.0	0.0
<i>B. aerogenes</i>	0.25	1.0	3.0	5.0
<i>B. cloacae</i>	0.5	1.5	2.0	3.0
<i>B. acidl.</i>	0.0	0.0	0.0	0.0
<i>B. paratyphosus A.</i>	0.0	?	?	?
<i>B. paratyphosus B.</i>	0.0	0.0 ?	0.0 ?	?
<i>B. pullorum</i>	0.0	0.0	0.0	0.0
<i>B. sanguinarium</i>	0.0	0.0	0.0	0.0
<i>B. typhosus</i>	0.0	0.0	0.0	0.0
<i>B. dysenteriae</i> (Shiga).....	0.0	0.0	0.0	0.0
<i>B. abortus</i> (Bang).....	0.0	0.0	0.0	0.0
<i>B. abortus</i> (Wis.).....	0.0	0.0	0.0	0.0
<i>B. hoffmanni</i>	0.0	0.0	0.0	0.0
<i>B. diphtheriae</i> (Park No. 8).....	0.0	0.0	0.0	0.0
<i>B. diphtheriae</i> (Y. M. S.).....	0.0	0.0	0.0	0.0
<i>V. cholerae</i> (R).....	0.0	0.0	0.0	0.0
<i>V. cholerae</i> (Y. M. S.).....	0.0	0.0	0.0	0.0
<i>Sarcina lutea</i>	0.0	0.0	0.0	0.0
<i>Sarcina aurantiaca</i>	0.0	0.0	0.0	0.0
<i>Mic. tetragenus</i>	0.0	0.0	0.0	0.0
<i>Mic. flavus</i>	0.0	0.0	0.0	0.0
<i>Staph. aureus</i>	0.0	0.0	0.0	0.0
<i>Staph. albus</i>	0.0	0.0	0.0	0.0
<i>Str. pyogenes</i> (309).....	0.0	0.0	0.0	0.0
<i>Str. pyogenes</i> (346).....	0.0	0.0	0.0	0.0
<i>Streptothrix</i>	0.0	?	?	+
<i>Cladothrix</i>	0.0	+	+	++

In order to determine whether the presence of taurin and creatin exert an inhibitive influence glyocoll was added to these mediums. The results were essentially the same as when glyocoll alone was added to the substratum. Taurin and creatin are, therefore, merely indifferent, or resistant to bacterial action in the concentrations employed (0.1%).

The purins hypoxanthin and uric acid were next employed, as was also allantoin, an oxidation product of uric acid. The results obtained with hypoxanthin and uric acid were comparable, as might be anticipated, owing to their

similarity in structure. Of the two agents the uric acid assumes the more importance, because certain differences which are brought about in both of these mediums are most accentuated by the uric acid. Table 7 presents the results of the uric acid experiments. It will be seen that certain organisms which grew in the amino-acid mediums failed to attack the uric acid, or at the most underwent but slight development in its presence. These are the subtilis and the proteus groups, *B. coli*, *B. acidi lactici*, the paratyphoid bacilli, *B. sanguinarium* and the cholera vibrio. The uric acid and hypoxanthin are inferior, therefore, to the amino-acids as sources of available nitrogen.

The points of special significance in the uric acid table is the sharp differentiation between the reactions of *B. coli* and *B. acidi lactici* on the one hand, and *B. aerogenes* and *B. cloacae* on the other. The former leave the medium unclouded, whereas *B. aerogenes* and *B. cloacae* produce luxuriant growths. In view of the recent interest attached to these types of organisms a more extended investigation was conducted on this method of their differentiation. The results have been published elsewhere.¹ Further experimentation has confirmed the earlier results, and the value of the uric acid synthetic medium in the separation of the coli and the aerogenes types of organisms appears to us to have been fully established. The results obtained with the uric acid medium are definitely correlated with the methyl red and the Voges-Proskauer reactions.

As uric acid is insoluble in water, but soluble in alkalies, it was necessary to omit the primary potassium phosphate from the constant substratum. By this procedure a 0.1% solution of uric acid, or rather potassium urate, could be obtained.

Although structurally quite similar to uric acid, allantoin yields entirely different results, deporting itself in about the same manner as the amino-acids. Presumably the free amino grouping in the allantoin renders this substance a more acceptable source of nitrogen than the purins in which all of the nitrogen is held in the purin ring.

COMBINATIONS OF TWO OR MORE OF THE FOREGOING NITROGENOUS SUBSTANCES

The chemically-definite mediums employed up to this point contained only one nitrogen-containing ingredient, with the single exception of the Uschinsky solution. In the following experiments the culture medium was enriched by one or more additional nitrogenous substances. It was assumed that such combinations would tend to satisfy the nitrogen requirements of bacteria better than when the choice of nitrogen is more limited.

The first combination was one of glycocoll, aspartic acid and tyrosin. The same organisms were employed in this and subsequent experiments as heretofore. Contrary to expectations, the results were in nowise different from those obtained with the single nitrogenous substances, as for example glycocoll.

A combination of aspartic acid and lysin, or of aspartic acid, lysin and histidin, likewise proved to be of no added advantage.

In the next test creatin was added to a medium which already contained glycocoll, lysin and histidin. A careful comparison of the results with those obtained with the single nitrogenous substances showed them to be practically identical.

Finally, a mixture of the following agents was used: glycocoll, 0.05%; valin, 0.05%; aspartic acid, 0.05%; glutamic acid, 0.05%; tyrosin, 0.03%; a trace of lysin dichlorid; histidin monochlorid, 0.07%; tryptophan, 0.05%;

¹ Koser, S. A.: Jour. Infect. Dis., 1918, 23, p. 377.

taurin, 0.05%; creatin, 0.05%, and allantoin, 0.05%. The resultant solution (in the constant substratum described in the early part of this paper) was rendered neutral to litmus by the addition of normal sodium hydroxid. The results are presented in Table 8.

TABLE 8

CONSTANT SUBSTRATUM PLUS MIXTURE OF 11 DIFFERENT NITROGENOUS SUBSTANCES
(SEE PAGE ?)

	Degree of Growth at Expiration of			
	24 Hrs.	48 Hrs.	4 Days	7 Days
<i>B. cereus</i>	+	++	+++	+++
<i>B. subtilis</i>	+	++	++	++
<i>B. ramosus</i>	+	++	++	++
<i>B. anthracis</i>	0.0	0.0	0.0	0.0
<i>B. fluorescens</i>	1.0	2.0	2.0	2.0
<i>B. fluorescens nonliq.</i>	0.75	3.0	4.0	4.0
<i>B. pyocyaneus</i>	1.5	1.5	1.0	1.0
<i>B. prodigiosus</i>	2.0	2.0	3.0	4.0
<i>B. ruber</i>	1.0	2.0	3.0	4.0
<i>Proteus vulgaris</i>	0.25	0.25	0.25	0.25*
<i>Proteus mirabilis</i>	0.25	0.5	0.5	0.5 *
<i>Proteus zenkeri</i>	?	?	0.25	0.25†
<i>B. capsulatus</i>	1.5	1.5	3.0	5.0
<i>B. coli</i> (104).....	1.5	4.0	5.0	5.0
<i>B. coli</i> (U).....	0.0	0.0	2.0	3.0
<i>B. aerogenes</i>	2.0	4.0	5.0	7.0
<i>B. cloacae</i>	1.5	4.0	3.0	5.0
<i>B. acidi lactici</i>	2.0	3.0	4.0	5.0
<i>B. paratyphosus A</i>	0.5	0.75	0.75	0.75
<i>B. paratyphosus B</i>	0.5	0.5	0.75	1.0
<i>B. pullorum</i>	0.25	0.25	0.25	0.25
<i>B. sanguinarium</i>	0.5	1.0	1.0	1.0
<i>B. typhosus</i>	0.5	0.5	0.5	0.75
<i>B. dysenteriae</i> (Shiga).....	0.0	0.0	0.0 ?	0.0 ?
<i>B. abortus</i> (Bang).....	0.0	0.0	0.0	0.0
<i>B. abortus</i> (Wis.).....	0.0	0.0	0.0	0.0
<i>B. hoffmanni</i>	0.0	0.0	0.0	0.0
<i>B. diphtheriae</i> (Park No. 8).....	0.0	0.0	0.0	0.0
<i>B. diphtheriae</i> (Y. M. S.).....	0.0	0.0	0.0	0.0
<i>V. cholerae</i> (R).....	0.5	0.5	0.75
<i>V. cholerae</i> (Y. M. S.).....	0.25	1.0	1.5
<i>Sarcina lutea</i>	0.0	+	++	++
<i>Sarcina aurantiaca</i>	0.0	0.0	0.0	0.0
<i>Mic. tetragenus</i>	0.0	0.0	0.0	0.0
<i>Mic. flavus</i>	0.0	0.0	0.0	0.0
<i>Staph. aureus</i>	0.0	0.0	0.0	0.0
<i>Staph. albus</i>	0.0	0.0	0.0	0.0
<i>Staph. citreus</i>	0.0	0.0	0.0	0.0
<i>Str. pyogenes</i> (U).....	0.0	0.0	0.0	0.0
<i>Streptothrix</i>	0.0	+	++	++
<i>Cladothrix</i>	0.25	0.5	++	++

* Deep yellow.

† Failed to develop when re inoculated into 2nd tube.

The development in this very complex medium of those organisms which grow in the simple nitrogenous mediums is somewhat accelerated, especially within the first 24-48 hours. Furthermore, *B. typhosus*, *B. pullorum* and *Sarcina lutea* were able to multiply in this medium. These organisms were carried through several successive cultures with no apparent diminution of growth. Several strains of *B. typhosus* were used, all giving the same results.

Proteus zenkeri exhibited a very slight growth in the first culture, but was unable to develop when transferred to a second tube. In every instance where histidin was present *Proteus vulgaris* and *Proteus mirabilis* produced the peculiar yellow color that has previously been mentioned. It is to be noted that in this complex medium, as in the simpler solutions, *B. anthracis*, *B. abortus*, *B. diphtheriae*, *B. Hoffmanni* and all but one of the coccoid forms are unable to develop.

DISCUSSION

A general survey of the present investigation leads to the conclusion that the various amino-acids are quite similar in their ability to support the growth of certain micro-organisms. We see that certain species appear to be able to utilize and to synthesize their own protoplasm as readily from a very simple amino-acid, glycocoll, as from histidin or tryptophan. On the other hand, a number of organisms, as *B. abortus* and *B. diphtheriae*, are evidently unable to assimilate directly any of the amino-acids, extractives, or other sources of nitrogen which were offered.

The aerobic spore-formers, *B. cereus*, *B. subtilis* and *B. mycoides*, develop rather scantily and slowly in the amino-acid mediums. At times the growth is irregular and may fail to appear. The anthrax bacillus refused to grow in every case. The two species of fluorescent bacilli, *B. pyocyaneus*, *B. prodigiosus* and *B. ruber balticus*, develop readily when any one of the amino-acids, diammonium acid phosphate, hypoxanthin, uric acid, or allantoin, is supplied as the only source of nitrogen. The colon bacillus and closely allied organisms are also able to utilize the amino-acids, ammonium acid phosphate, urea and allantoin. *B. capsulatus*, *B. cloacae* and *B. aerogenes* apparently can attack hypoxanthin and uric acid, whereas the colon bacillus lacks this power. The paratyphoid bacilli appear to be quite "cosmopolitan" in their nitrogen requirements, for they greatly resemble *Bacillus coli*, although the growth is not as luxuriant. *B. dysenteriae* refused to grow in any of the mediums, while *B. typhosus* was able to develop to a slight extent in several instances only, that is, in tryptophan and several amino-acid combinations.

In view of the striking cultural resemblance of *Bacterium pullorum* to *Bacterium sanguinarium*, it is interesting to observe that the latter organism is much less exacting in its nitrogen requirements than the other. *Bacterium sanguinarium* developed on amino-acids, diammonium acid phosphate, and allantoin.

The proteus organisms exhibited considerable irregularity. At best their development was meager. Negative results were obtained after

the introduction of limited numbers of cells. This leads one to the conclusion that the power of these organisms to utilize directly the amino-acids is exceedingly slight. *Proteus zenkeri*, which apparently is in no way related to the above forms (Wenner),* failed to grow in all instances.

The two strains of the cholera vibrio appear to be less exacting than the majority of the pathogenic species herein employed, for they utilized asparagin and any one of the amino-acids, although not the other sources of nitrogen. *B. abortus*, *B. diphtheriae* and *B. hoffmanni* exhibited no evidence of multiplication in any instance. The same may be said of all but one of the cocci studied.

By multiplying the sources of nitrogen (Table 8) it was found that most of the organisms which developed exhibited a more luxuriant growth than on the more simple mediums, especially within the first 24-48 hours. The various miscellaneous sources of nitrogen appear to be inferior to the amino-acids, although the diammonium acid phosphate and allantoin gave almost as good results.

No attempt was made to study pigment production. Incidentally, it was observed that cultures of *B. pyocyaneus* presented, as a rule, the characteristic deep green shade. Both types of *B. fluorescens* exhibited a light yellowish-green color, while the cultures of *B. prodigiosus* and *B. ruber balticus* failed to show the characteristic red pigment.

The demonstration that those organisms which are able to develop on amino-acid mediums are still enabled to do so when only a few hundred cells are introduced into each tube is especially interesting in that it indicates a power on the part of these organisms to utilize directly the amino-acids. In view of the work of previous investigators who have shown that bacteria cannot directly attack native proteins, and in some instances not even proteoses or peptones, this fact is particularly significant. It would appear that some species, at least, find immediately available nitrogen in the form of isolated amino-acids or of the other chemically-definite nitrogenous bodies employed throughout the course of this work.

It will be remembered, however, that some micro-organisms showed no indication of development on any of these substances. It is interesting to speculate as to what may be the probable nature of the nitrogenous bodies required by these species for initial development.

* Doctorate thesis, 1918, Yale University Library.

It may be found that dipeptid or more complex polypeptids are necessary, although such evidence as exists would seem to point in the opposite direction.

Lloyd (1916) and others have suggested the necessity of "vitamins" or "accessory growth factors" for the initial cultivation of the meningococcus. The same idea has been advanced by several other writers. This opens a new field in the study of bacterial nutrition. Investigation in this field is beset with difficulties, however, since very small amounts of chemically definite nitrogenous substances may suffice to initiate bacterial development, independent of "vitamins."

A third possibility suggests itself. The nitrogen which the more "exacting" pathogens first seize on may be contained in very simple and unstable bodies which defy all methods of isolation. It seems probable that the usefulness of blood or serum, when added to ordinary culture mediums, is due to the presence of substances of this nature, substances which are readily attacked and which may pave the way, so to speak, for the attack on more complex nitrogenous bodies.

SUMMARY

All of the amino-acids employed seem to form a rather broad group in so far as their ability to furnish nitrogen to the organisms in question is concerned. Tryptophan may be a possible exception to this.

Asparagin, the diamino-acid lysin, and the heterocyclic amino-acid histidin, apparently possess no advantage over the mono-amino acids.

A number of organisms were shown to be capable of passing through 12 successive cultures in an amino-acid medium without any diminution in the luxuriance or rapidity of development.

Those organisms which find an amino-acid medium suited to their needs are able to develop, at least to a slight extent, when very small quantities of amino-acid are supplied.

If only a few hundred cells be introduced into the amino-acid culture medium, multiplication takes place as usual and eventually the growth attained in these cultures is as luxuriant as that resulting from a much larger inoculum.

When glycerol is withdrawn from the chemically-definite mediums the bacterial development is not so luxuriant. Dextrose can be substituted for glycerol.

With the exception of the cholera vibrio, the organisms which utilize an amino-acid can as readily initiate development on diammonium acid phosphate as on the amino bodies.

Urea, taurin, creatin, hypoxanthin, and uric acid are inferior to the amino-acids as immediately available sources of nitrogen. Allantoin gives results which are quite comparable to those of the amino-acids.

Combinations of amino-acids, or of amino-acids and other nitrogen-containing compounds apparently possess little value over any one of the single amino-acids.

In a medium consisting of uric acid, together with certain mineral salts and glycerol, *Bacillus aerogenes* grows luxuriantly while the colon bacillus is unable to develop.

B. anthraxis, *Proteus zenkeri*, *B. abortus*, *B. diphtheriae*, *B. hoffmanni*, *B. dysenteriae*, and all of the cocci studied — with the exception of *Sarcina lutea* in a few cases — consistently failed to develop in all of the mediums employed. *Bacterium pullorum* developed slightly in one instance only, while *B. typhosus* exhibited a slight growth in a few mediums.

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A COMPARISON, WITH THE STANDARD PLATE METHOD, OF SOME RAPID METHODS FOR BACTERIOLOGIC ANALYSIS OF MILK *

J. E. SIMMONS

Agricultural Experiment Station, University of Wisconsin, Madison, Wis.

The bacterial content of a sample of milk is one of the most important of the many factors that determine the past history of the milk and gives some idea as to its future with reference to keeping quality. To control the quality of the milk supply which the city milk dealer receives, it is very desirable that he should know the bacterial content of the milk from each patron. He should also know the bacterial content of the milk as it leaves each piece of machinery in the milk plant. If this information is obtained, the dealer will know the kind of product which he is placing on the market for consumption. Since milk dealers handle large quantities of milk, the determination of the bacterial content is an enormous task. It would be desirable to use some method of analysis which is more rapid, less expensive of time and equipment than the "standard plate method." It was the purpose of this investigation to compare some of the simpler and quicker methods with the standard plate method in order to determine their practical value.

HISTORICAL REVIEW

The first method used to count bacteria in milk was by means of the microscope.¹ The difficulty which was encountered in this method was in the preparation of the material for examination. It was almost impossible to obtain an even distribution of the milk so that the organisms could be easily distinguished and counted. This difficulty rendered accurate counts impossible at first and the results obtained were only approximately correct.

With the introduction of the plate method by Koch about 1880, the microscopic method of estimating bacteria in milk was almost completely abandoned. The plate method has been adopted as the standard method of milk analysis by the American Public Health Association.²

Within the last few years several methods have been devised which have not been widely adopted but are promising. These methods have been devel-

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¹ Hueppe, Ferdinand: *Die Methoden der Bakterien-Forschung*, 1891.

² *Standard Methods for the Bacteriological Analysis of Milk*, 1916.

oped because of certain disadvantages of the standard plate method. Chief among these disadvantages are: (1) the length of time required for incubation, 48 hours being the shortest; (2) large amount of glassware and culture medium required, as well as other expensive equipment, and (3) the paucity of details regarding the kinds of organisms present.

About 1900, attention was again called to the microscopic method of counting bacteria in richly seeded material. Klein,³ Winslow,⁴ and others have worked on this method with good results. Slack,⁵ in 1906, introduced a method in which he sought to determine the bacterial content of milk by counting the bacteria in the sediment obtained by centrifugation. Studying a large number of samples, he worked out an arbitrary ratio between the number of bacteria in the field of the microscope and the results obtained by the plate counts. Results obtained by this method in the hands of others do not appear to be satisfactory. The reasons for this were the personal equation of individual workers, speed and length of centrifugation, etc.

In 1911, Breed⁶ introduced a direct microscopic method which has been used quite extensively. In his method 0.01 cc of milk was spread over 1 sq. cc, stained and examined directly under the microscope. Breed claimed that the number of bacteria determined by this method was quite comparable to the number determined by plate counts, if clumps instead of individual bacteria were counted. This method has the disadvantage of not being accurate with pasteurized milk or milks having low bacterial counts. However, if a rapid examination of raw milk was desired, this method could be used to determine both the number and character of the bacteria present with a fair degree of accuracy.

The reduction of dyes by bacteria in milk has been observed for some time. Duclaux⁷ was probably the first to work on the reduction of dyes with special reference to milk. Since then, there have been quite extensive studies made of this method by Jensen,⁸ Fred,⁹ Barthel,¹⁰ and others. All of these workers arrived at practically the same conclusions; namely, that (1) in a general way the reduction time is inversely proportional to the bacterial content; (2) that nearly all bacteria reduce dyes, and (3) that methylene blue gives the best and most uniform results. In order to obtain consistent results, it is essential (1) that the dye substance have a known formula; (2) that the strength of the solution used be constant; (3) that the solution be dilute enough not to interfere with the development of the micro-organisms, and (4) that the change in the appearance of the milk during reduction be quite pronounced. By comparing the time of reduction with the number of bacteria obtained by the plate method, a classification was formulated which can be used to correlate the reduction test with the bacterial count. Fred⁹ has given milks the following rating: If reduction occurred in from 7-30 hours, the milk contains 1,000-500,000 bacteria per cc; in 2-7 hours, from 500,000-2,000,000; in one-quarter to 2 hours, about 15,000,000, and less than

³ Centralbl. f. Bakteriologie, II, 1900, 7, p. 834.

⁴ Jour. Infect. Dis., 1905, Suppl. 1, p. 209.

⁵ Centralbl. f. Bakteriologie, II, 1906, 16, p. 537.

⁶ Centralbl. f. Bakteriologie, II, 1911, 30, p. 337.

⁷ Le Lait, Paris, 1894, p. 5.

⁸ Centralbl. f. Bakteriologie, II, 1908, 18, p. 211.

⁹ Centralbl. f. Bakteriologie, II, 1912, 35, p. 391.

¹⁰ Ztschr. f. Untersuch. d. Nahrungs- u. Genussmittel, 1911, 21, p. 513.

one-fourth hour from 10,000,000-150,000,000. While the test was only very roughly quantitative, it was a rapid way of determining whether a milk was high or low in bacterial content. Its chief advantage lies in the fact that the test is easily made and is quite rapid.

Each of the foregoing methods has its advantages which make its use possible in some place, either in the creamery, routine laboratory, or in research work. But each also has some disadvantages which prevent it from being entirely satisfactory. Most of the rapid methods at the present time are either roughly quantitative tests or are not applicable to all kinds of milk. It would seem that a method to be generally useful should be accurate, economical of time and money, and equally applicable to all milks. Frost,¹¹ in 1915, devised a method of determining the number of bacteria in milk which promises to be used quite extensively in the future. This method will be referred to as the "little plate" method in this text. Several advantages have been claimed for this method. Among these are: (1) rapidity, requiring only 4-8 hours to complete the analysis; (2) reasonable accuracy; (3) small amount of glassware and medium; (4) a permanent record, and (5) a differentiation of various types of bacteria present.

Up to the present time no quantitative work has been published on this method except by Frost. He has made comparative analyses on a considerable number of milks of varying bacterial content. The results appeared to correspond reasonably close with those obtained by the standard plate method and were obtained in about one-eighth the time. In addition to the quantitative count obtained, important information could be gained by studying the colony formation.

METHODS USED

In this study a series of milks was examined by making parallel counts by five methods of analysis, namely, direct microscopic; standard plate; lactose agar plate; little plate, and reduction test. The bacterial count obtained by the standard plate method, as recommended by the Committee on Milk Analysis appointed by the American Public Health Association, was used as a basis for comparison. Several dilutions were always made in order to have plates with the proper number of colonies. The plates were counted with a lens which magnified $3\frac{1}{2}$ diameters. Plates were also made on a medium containing 1% lactose.

The direct microscopic plate was made by spreading 0.01 c.c. of milk evenly over an area of 1 sq. cm. on a clean glass slide. This milk was measured with an accurately calibrated pipet. The preparation was dried and, after the fat had been removed with xylol, it was fixed and stained. The number of groups or clumps of bacteria was counted, using the oil-immersion lens. The

¹¹ Science, N. S., 1915, 42, p. 255. Jour. Infect. Dis., 1916, 19, p. 273.

size of the microscopic field, for the combination of lenses used, was compared with the area of the smear. This gave a factor of 526,000. The average number of clumps in a field multiplied by this factor gave the bacterial content per c c.

In the reduction test the following formula was used:

Methylene blue—med. pur.....	1 gm.
Sodium chlorid.....	8.5 gm.
Distilled water.....	1,000 c c

One c c of this solution was put in a sterile test tube with 10 c c of the milk to be analyzed. The two were thoroughly mixed and then placed in the incubator at 37° C. and the length of time required to decolorize the methylene blue was noted. Numerous investigators have recommended that the methylene blue solution should be sterile, but tests were made using sterile and non-sterile methylene blue, and no appreciable difference in the reduction time could be noticed. The methylene blue was always measured, however, with a sterile pipet. No oil was placed on the surface of the milk in the test tubes as the cream came to the surface in a few minutes, thus partially excluding the air and preventing oxidation of the dye. The oil was necessary only when the tubes were observed at infrequent intervals. Frequent observations were made after the tubes were placed in the incubator.

In the little plate method, 1 c c of the sample of milk to be analyzed was mixed thoroughly with 1 c c of nutrient agar which had previously been liquefied and cooled to 45 C. In cases in which the milk was known to have a high bacterial content, the milk was diluted 10 times with sterile water before mixing with the liquefied agar. One-tenth of a c c of the mixture was then spread evenly over an area of 4 sq. cm. which had previously been marked off on a clean glass slide. The slide was flamed immediately before use to prevent contamination. When the agar had solidified, the slide was placed in a moist chamber and incubated at 37° C. The period of incubation was from 5-6 hours with good milk. At the end of the incubation period, the slides were removed and dried quite rapidly at nearly 100° C. on a hot plate. While the slides were still hot, they were immersed in a solution of 10% acetic acid in 95% alcohol for at least 1 minute. Without washing, the slides were immersed for 3-5 minutes in a staining solution, made by diluting 1 part of Loeffler's methylene blue with 4 parts of distilled water. This stained the colonies deep blue, while the background was light blue or not stained at all. The slides were then washed thoroughly and dried over a warm plate. The area of the microscopic field was determined for the low, high and oil immersion lenses. When the lower power was used, one colony per microscopic field represented 4,540 bacteria per c c; under high power, one colony per field represented 93,000 bacteria per c c, and under oil immersion, one colony per field represented 421,000 bacteria per c c. If the milk was diluted 1:10 before mixing with the agar, the above figures were multiplied by 10. The counting was done with the lens which gave the easiest field to count and in most cases this was the low power. With some of the good milks the entire plate was examined in order to get an accurate count.

SOURCES OF MILKS

The milks used were obtained from several sources so that the bacterial contents varied widely. This was desirable in order to determine if the rapid methods were applicable to all grades of milk. High grade milks were

obtained at the university dairy barn (D. B.). Many samples were obtained from the university creamery (U. C.). The milk received at this place was representative of milk produced on dairy farms where no special care was used to produce a product of high quality. A few samples were obtained from dealers.

The pasteurized milks were obtained from local milk distributors (K. M. and Z.). A number of milks were also pasteurized in the laboratory (L.). This was done in small sterile bottles holding about 150 c.c. A thermometer was inserted into the bottle so that the bulb was in the center of the milk. The milk was held at 62.5° C. for 30 minutes after the thermometer in the milk showed this temperature. In some cases, the samples were analyzed immediately, while others were placed in the icebox until the following day.

EXPERIMENTAL DATA

The milks analyzed have been divided into 4 classes according to the number of bacteria they contained as determined by the standard plate method. Class A consists of those containing less than 10,000 bacteria per c.c.; Class B, those containing between 10,000 and 100,000 per c.c.; Class C, those containing between 100,000 and 1,000,000 per c.c., and Class D, those containing in excess of 1,000,000 per c.c.

TABLE 1
CLASS A MILKS

Serial No.	Kind of Milk	Source	Number Bacteria per C C on				Reduction Time in Hours
			Breed Plate	Standard Plate	Lactose Plate	Little Plate	
1	Past.	L.	500,000	50	260	60	30
2	Past.	L.	500,000	100	170	120	30
3	Past.	L.	850,000	100	200	275	30
4	Past.	L.	500,000	200	100	80	30
5	Past.	L.	500,000	400	800	275	30
6	Past.	L.	500,000	800	1,700	225	30
7	Past.	L.	500,000	1,000	1,000	140	30
8	Raw	U. C.	500,000	1,000	2,000	1,200	24
9	Past.	L.	500,000	1,300	1,400	700	30
10	Past.	L.	Innumerable	1,600	2,700	4,900	18
11	Raw	U. C.	500,000	1,700	1,500	30
12	Past.	L.	500,000	2,000	2,500	1,600	24
13	Raw	D. B.	500,000	2,100	1,900	17
14	Raw	D. B.	500,000	2,500	4,600	3,500	3
15	Past.	L.	500,000	2,700	11,000	9,100	30
16	Past.	L.	1,100,000	3,400	5,600	4,500	30
17	Past.	L.	831,500	3,400	2,500	2,200	30
18	Raw	D. B.	500,000	5,000	3,000	27
19	Raw	U. C.	500,000	5,000	5,000	6,400	15
20	Past.	L.	44,000,000	5,100	4,900	2,200	30
21	Past.	M.	500,000	6,000	7,000	5,500	22
22	Past.	L.	2,000,000	6,000	3,700	4,500	30
23	Raw	U. C.	500,000	8,800	4,600	5,400	10

From the foregoing table it can be seen that comparatively few counts were obtained by the direct microscopic method in this group of milks. Any figures given for milks with counts less than 600,000, or one bacterium per microscopic field, are only rough approximations.

The other figures given are for pasteurized milks and include dead bacteria and therefore show no relation to the results obtained by the cultural methods.

In the lactose agar plates, the counts obtained were higher 12 times, lower 6 times and twice alike. Averaging the counts obtained by the 2 methods, it will be found that the increase due to the lactose was approximately 20%.

TABLE 2
CLASS B MILKS

Serial No.	Kind of Milk	Source	Number Bacteria per C C on				Reduction Time in Hours
			Breed Plate	Standard Plate	Lactose Plate	Little Plate	
24	Past.	L.	38,900,000	13,000	18,900	29,000	30
25	Past.	M.	5,500,000	13,000	6,500	9,500	30
26	Raw	U. C.	500,000	15,000	30,000	31,400	13
27	Past.	L.	3,500,000	17,000	22,300	13,500	16
28	Past.	L.	Innumerable	17,000	10,200	18,200	30
29	Raw	D. B.	500,000	18,000	48,000	20,400	29
30	Past.	L.	69,000,000	18,300	65,000	54,000	30
31	Raw	U. C.	500,000	19,000	17,000	23,600	16
32	Raw	U. C.	500,000	22,000	26,000	24,600	14
33	Raw	U. C.	500,000	22,000	32,000	13,600	15
34	Raw	U. C.	16,000,000	23,000	35,000	22,700	12
35	Raw	U. C.	500,000	24,000	27,000	30,100	12
36	Raw	Z.	3,000,000	25,000	27,000	22,700	14
37	Raw	U. C.	500,000	25,000	27,000	27,700	12
38	Raw	U. C.	500,000	25,000	42,000	56,000	10
39	Raw	Z.	7,360,000	30,000	57,000	27,200	15
40	Raw	U. C.	500,000	30,000	68,000	18
41	Past.	L.	Innumerable	30,800	50,000	132,000	20
42	Past.	M.	Innumerable	31,000	86,000	200,000	18
43	Past.	M.	2,800,000	35,000	35,000	23,600	24
44	Raw	U. C.	500,000	35,000	21,000	22,600	15
45	Raw	U. C.	500,000	37,000	47,000	50,000	15
46	Raw	U. C.	500,000	37,000	40,000	28,000	12
47	Past.	L.	Innumerable	41,000	41,000	45,500	30
48	Raw	U. C.	500,000	41,000	133,000	91,000	10
49	Raw	U. C.	500,000	41,000	86,000	127,000	12
50	Raw	U. C.	500,000	44,000	63,600	15
51	Raw	K.	6,300,000	45,000	54,000	91,000	15
52	Past.	Z.	3,200,000	46,000	40,000	42,600	16
53	Raw	U. C.	500,000	46,000	99,000	132,000	12
54	Past.	Z.	11,000,000	52,000	12,000	29,000	24
55	Raw	U. C.	500,000	53,000	31,000	53,000	15
56	Past.	Z.	7,900,000	55,000	98,000	63,000	20
57	Past.	K.	24,000,000	57,000	90,000	54,000	22
58	Raw	D. B.	500,000	64,000	28,600	22
59	Raw	U. C.	500,000	64,000	75,000	55,800	4
60	Raw	U. C.	500,000	66,000	107,000	59,900	8
61	Raw	U. C.	500,000	69,000	91,000	77,100	12
62	Past.	Z.	3,200,000	72,000	34,000	54,500	20
63	Past.	L.	3,200,000	77,000	55,000	59,000	30
64	Past.	K.	1,300,000	79,000	11,000	68,100	30
65	Raw	D. B.	500,000	80,000	28,100	22
66	Raw	D. B.	500,000	80,000	26,800	24
67	Raw	U. C.	500,000	80,000	89,000	254,500	9
68	Raw	U. C.	500,000	87,000	67,000	115,300	10
69	Raw	U. C.	500,000	95,000	115,000	493,000	15
70	Past.	Z.	3,700,000	97,000	79,000	100,000	18

A long period of time was required to decolorize the methylene blue. If we disregard one specimen (Sample 14), which is evidently not comparable, the time varied from 10-30 hours, 17 out of the 23

samples required 24 hours or more. This test is apparently much more reliable than the Breed method for this class of milks, and the results would compare favorably with the standard plate figures. The little plate method gave results which are closely comparable to the standard plate count. Nine of the counts were higher on the little plates, while 14 were lower.

As in the preceding table, there were too few bacteria on the slide to be counted accurately by the direct microscopic method. The other figures given were all of pasteurized milks and have no direct relation to the living organisms.

The lactose agar plates again gave an increased count. The plates were higher 28 times and lower than the standard plates 12 times. The average increment due to the lactose was about 16%.

Thirty-nine of the 47 samples were put in the same class by the little plate and standard plate methods. Of the 8 put in different classes, 2 (Samples 68 and 70) were very close to the dividing line, hence were closely comparable. Twenty-six of the counts were higher on the little plates than on the ordinary plate method, while 20 of them were lower. One specimen (Sample 55) gave a similar count by the 2 methods.

The time required for the reduction of the methylene blue, excluding one sample (Sample 59), varied from 8-30 hours. Forty-one of the 47 samples in this class required 12 hours or more.

Nine of the samples counted by the direct microscopic method were raw milks and the figures obtained for these compare favorably with the standard plate counts in 5 cases. As in the previous tables, none of the 14 pasteurized milks counted gave comparable counts.

The lactose plates again gave larger numbers than the plain agar, the increased count being slightly over 20%. It is worthy of notice, however, that this average increase was due to the marked increase of a few samples for 22 of the milks gave a lower count than the standard plates and only 12 higher.

Of the 38 samples in this class, 31 of them were put in the same class by the little plate and ordinary plate method. Of the 6 put in different classes, all were quite closely comparable and were very much closer than duplicates on ordinary plates.

The average methylene blue reduction time was much shorter than in the preceding classes. The time varied from 8-30 hours, while 22 of the 38 samples required between 7 and 12 hours.

TABLE 3
CLASS C MILKS

Serial No.	Kind of Milk	Source	Number Bacteria per C C on				Reduction Time in Hours
			Breed Plate	Standard Plate	Lactose Plate	Little Plate	
71	Raw	U. C.	9,500,000	100,000	109,000	63,000	12
72	Past.	L.	4,600,000	101,000	94,000	218,000	16
73	Raw	U. C.	500,000	102,000	122,000	363,000	8
74	Raw	U. C.	500,000	103,000	384,000	213,000	10
75	Past.	K.	6,300,000	110,000	51,000	183,000	15
76	Raw	U. C.	500,000	118,000	117,000	114,000	9
77	Raw	U. C.	500,000	125,000	115,000	194,000	10
78	Past.	L.	Innumerable	126,000	121,000	63,200	20
79	Raw	D. B.	500,000	127,000	180,000	15
80	Past.	K.	4,300,000	130,000	127,000	132,000	16
81	Raw	U. C.	500,000	132,000	251,000	76,000	12
82	Raw	U. C.	500,000	136,000	107,000	180,000	12
83	Raw	U. C.	500,000	150,000	250,000	228,000	8
84	Past.	Z.	8,400,000	160,000	210,000	100,000	18
85	Past.	M.	6,700,000	167,000	250,000	155,000	20
86	Past.	L.	42,000,000	175,000	150,000	240,000	22
87	Past.	K.	6,800,000	200,000	174,000	651,000	16
88	Past.	M.	2,900,000	210,000	100,000	145,000	16
89	Raw	U. C.	500,000	215,000	96,000	12
90	Past.	M.	6,300,000	220,000	116,000	153,000	24
91	Raw	U. C.	500,000	232,000	174,000	400,000	8
92	Past.	M.	5,800,000	234,000	216,000	218,000	16
93	Raw	U. C.	2,100,000	250,000	800,000	960,000	8
94	Past.	K.	4,500,000	287,000	197,000	368,000	30
95	Raw	U. C.	500,000	290,000	267,000	324,000	10
96	Raw	U. C.	500,000	290,000	420,000	353,000	12
97	Raw	U. C.	500,000	327,000	395,000	359,000	10
98	Raw	U. C.	1,400,000	352,000	288,000	530,000	8
99	Past.	L.	15,800,000	390,000	680,000	744,000	16
100	Past.	K.	31,600,000	420,000	400,000	1,020,000	12
101	Raw	U. C.	5,900,000	470,000	390,000	459,000	7
102	Raw	U. C.	1,100,000	570,000	922,000	12
103	Raw	U. C.	1,600,000	613,000	555,000	1,000,000	10
104	Raw	U. C.	500,000	620,000	420,000	1,520,000	12
105	Past.	K.	4,200,000	650,000	520,000	482,000	18
106	Raw	U. C.	1,000,000	680,000	817,000	12
107	Past.	L.	32,000,000	680,000	2,100,000	2,410,000	26
108	Raw	U. C.	1,470,000	820,000	750,000	540,000	8

Twenty-five counts were made on the direct microscopic plate, and of these, 20 were quite closely comparable with the standard plates.

Less than half of these milks were plated on lactose agar, but the figures obtained were almost exactly double those on the standard plate method and 6 of the 8 samples gave higher counts. Of the 28 samples in Class D, only 4 were put in different classes by the standard plate and little plate method.

The reduction time was short in this group of milks varying from one-half hour to 10 hours. Eighteen of the 28 samples required 4 hours or less.

From the summary given in Table 5 it can be seen that the milks were placed in the same classes in nearly every case by the standard, lactose agar and little plate methods. Several of those which have been put in separate classes were very close, as they were very near the

division line between the two classes. No milks were put in Classes A or B with the direct microscopic method, as these classes contain so few bacteria per c c that an accurate count was impossible. A large number of the samples were put in Class D. These samples constituted the pasteurized milks and those whose bacterial content was very high. A considerable number of those put in Class D gave very close counts with the standard plates. A few of the Breed plates were seeded with clumps and single bacteria so that counts were impossible.

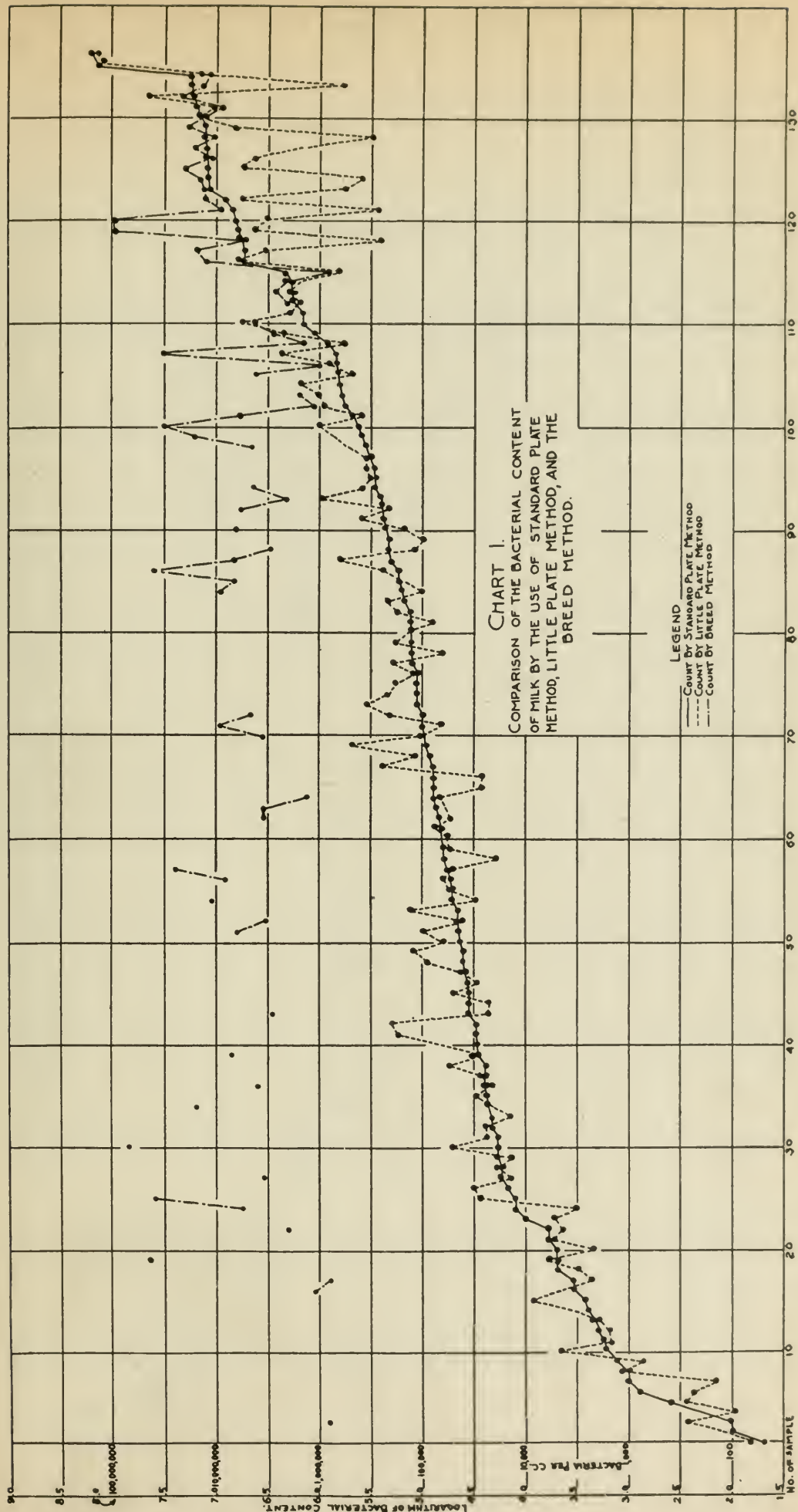
The data are shown also in Charts 1 and 2. The bacterial contents are represented by the logarithms of the numbers rather than by the actual numbers, which permits the plotting of all the different grades of milk in a single curve.

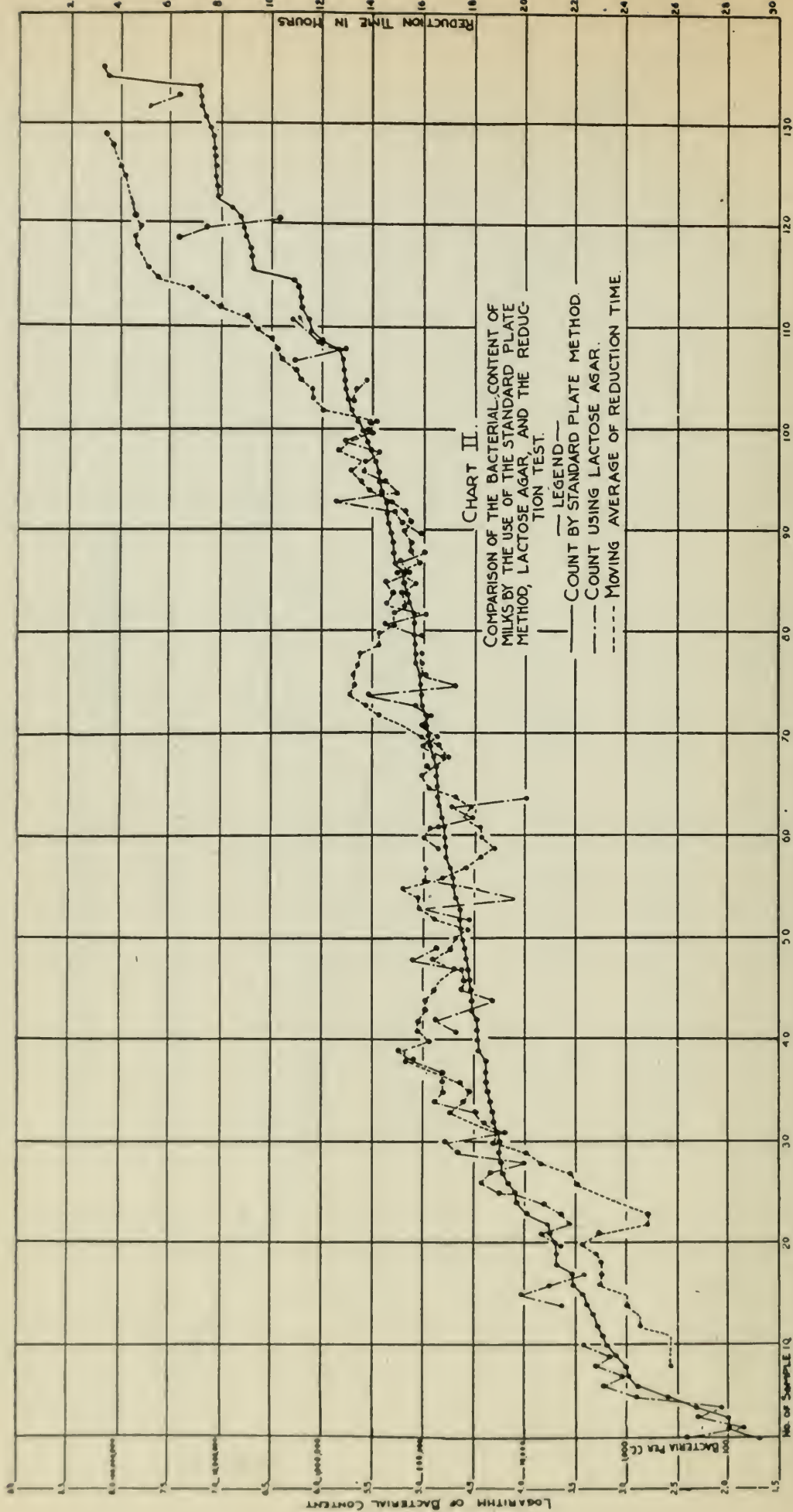
TABLE 4
CLASS D MILKS

Serial No.	Kind of Milk	Source	Number Bacteria per C C on				Reduction Time in Hours
			Breed Plate	Standard Plate	Lactose Plate	Little Plate	
109	Raw	U. C.	2,700,000	1,000,000	1,130,000	2,300,000	6
110	Raw	U. C.	4,700,000	1,400,000	1,600,000	5,900,000	4
111	Raw	U. C.	500,000	1,500,000	2,300,000	1,900,000	10
112	Raw	U. C.	2,100,000	1,700,000	1,670,000	4
113	Raw	U. C.	2,630,000	1,800,000	1,960,000	5
114	Raw	U. C.	2,100,000	1,840,000	1,500,000	8
115	Raw	U. C.	680,000	2,000,000	640,000	10
116	Raw	U. C.	12,600,000	5,300,000	6,300,000	3
117	Raw	U. C.	15,700,000	5,400,000	3,400,000	4
118	Raw	U. C.	5,400,000	5,600,000	2,500,000	4
119	Raw	U. C.	100,000,000	6,500,000	28,900,000	4,400,000	2
120	Raw	U. C.	100,000,000	6,600,000	12,800,000	3,200,000	3
121	Raw	U. C.	8,900,000	7,100,000	272,000	3
122	Raw	U. C.	13,500,000	8,400,000	3,000,000	6,000,000	6
123	Raw	U. C.	11,000,000	12,900,000	590,000	3
124	Raw	U. C.	13,700,000	12,900,000	376,000	2
125	Raw	U. C.	21,000,000	12,900,000	5,700,000	3
126	Raw	U. C.	11,000,000	13,100,000	4,130,000	8
127	Raw	U. C.	16,300,000	13,500,000	14,400,000	6
128	Raw	U. C.	10,000,000	13,900,000	2,860,000	2
129	Raw	U. C.	18,900,000	13,900,000	7,600,000	3
130	Raw	U. C.	14,200,000	14,600,000	14,880,000	5
131	Raw	U. C.	9,500,000	15,600,000	9,300,000	9
132	Raw	U. C.	61,500,000	16,700,000	60,000,000	48,000,000	2
133	Raw	U. C.	13,000,000	17,000,000	5,400,000	2
134	Raw	U. C.	12,300,000	17,200,000	9,100,000	13,700,000	2
135	Raw	U. C.	Innumerable	148,000,000	143,000,000	1 1/2
136	Raw	U. C.	Innumerable	160,000,000	155,000,000	1 1/3

TABLE 5
DISTRIBUTION OF MILKS INTO THE FOUR CLASSES BY THE VARIOUS METHODS USED

	Direct Microscopic Plates	Standard Plates	Lactose Plates	Little Plates
Class A.....	0	23	20	24
Class B.....	0	47	41	43
Class C.....	3	38	34	42
Class D.....	67	28	9	27
Total.....	70	136	104	136





The milks were arranged in an ascending series by the counts obtained on the standard plates and the results obtained by the other methods were indicated as variations from this.

In Chart 1 the direct microscopic count and the little plate count were compared with the standard plates. The curve representing the little plate followed the curve for the standard plate determinations throughout the course, while the direct microscopic curve was in nowise comparable until the germ content exceeded a million per c.c.

In Chart 2 a comparison was made of the lactose agar and the reduction test with standard plate method. The figures for the lactose and plain agar were represented by the logarithms as in Chart 1, but it seemed best to represent the reduction time by a "moving average."

The results of the reduction time in the tables appear to be very irregular, but when a large number of results were observed, there seemed to be quite a close correspondence between the reduction time and the standard plate counts. Since the variations in the reduction time appeared to be very irregular, a better idea of the comparison of this method with standard plates was obtained if the "moving average" of the reduction time was plotted, rather than the actual reduction time. King¹² says: "One of the best methods of smoothing certain varieties of histograms is to use a moving average to obtain a trend." The first step in the computation of the moving average was to obtain the average reduction time of the first 15 samples. This constituted the moving average for the eighth sample. By adding the sixteenth sample and dropping the first sample, the average can be found for the ninth sample. By successively dropping the first sample and adding one each time, the average could be determined to the end of the series. This method does away with single large fluctuations and made the two methods more comparable.

DISCUSSION

Breed Method.—The direct counts were not comparable with the counts by the other methods except with milks having quite a high bacterial content. When the bacteria were less than 500,000, the accuracy of the direct count was doubtful. However, the direct count served as a rapid means of determining whether a raw milk was high

¹² Elements of Statistical Method, 1912.

or low in bacteria. The application of the direct count to pasteurized milks does not seem feasible until a method has been devised to differentiate between dead and living bacteria.

The results obtained by the direct count are much higher than the standard plate counts on milks containing few bacteria. Several reasons for this difference are possible. Not all of the organisms in milk grow on plain agar. There may be some dead organisms in the milk which were counted under the microscope, but will not show on the standard plate. This was especially true in pasteurized milks.

While the correspondence between the direct count and the plate method was not close, the direct count served as an indication of the total number of bacteria regardless of whether they were active or not. It gave in a short time a general idea of the number of bacteria in the milk.

Lactose Agar.—While a good many of the counts on the lactose agar were lower than the corresponding count on plain agar, these variations were mostly within the range of experimental error. The same was not true of those having a count higher than on the plain agar, since many of the variations were 200 and 300% above those on plain agar.

It would thus seem that the lactose agar furnished a more suitable medium, especially for some milks. It also appeared that the colonies were considerably larger on lactose agar than they were on plain agar, so much so that a lens was not required in counting the lactose agar plates. This confirmed the findings of Sherman.¹³

Little Plate.—It can be readily seen that the correspondence between the little plates and the standard plates was quite close. The average count on the little plate was about 25% higher than on the standard plate. This higher count might be explained on the supposition that some bacteria in milk grew on the little plates but not on the standard plates. This was reasonable, since the little plates have a high percentage of milk in the culture medium. Milks having a high bacterial content appeared to give a lower count on the little plates than on the standard plates. This was possibly due to the fact that the clumps of bacteria were broken up when high dilutions were used in standard plates. This possibility of breaking up the clumps was very much less in the little plates, since only one dilution was necessary.

¹³ Jour. Bacteriol., 1916, 1, p. 481.

A careful study of the tables and charts also showed that there was a somewhat closer correspondence between the little plates and the lactose agar counts than there was between the little plates and the plain agar counts.

Reduction Test.—The reduction test, when used to measure the bacterial content of milk, showed considerable variation. However, it was a rapid and very easy method for determining the activity of the bacteria in the milk. While the time of reduction in this investigation appeared somewhat longer than that obtained by other workers, a correspondence does exist between the time of reduction and the number of bacteria. Additional tests not recorded here appear to show that the strength of the methylene blue solution can be reduced, thus making the time of reduction shorter. This is a matter of considerable practical value.

This test, in contrast to all other methods used to determine the bacterial content of milk, was very economical of equipment and very simple in technic.

SUMMARY

One hundred and thirty-six milks, varying in bacterial content from 50-160,000,000 per c c, have been analyzed by 5 different methods: Direct microscopic, standard plate, lactose plate, little plate (Frost), and reduction test.

In milks containing less than 1,000,000 bacteria per c c the 3 methods — standard plate, lactose plate and little plate — gave closely comparable results. The lactose plates on the whole gave results slightly higher than the plain agar plates, and these results seemed to follow the trend of the little plate counts somewhat more closely than the standard plates.

The direct microscopic count was less reliable with milks having a low bacterial content than it was with poor milks.

In milks containing upward of 1,000,000 bacteria per c c, the lactose plates were found to be considerably higher (50%) than the standard plates, the little plates tended to run somewhat lower, while the direct microscopic count gave highly satisfactory results.

The time of the reduction test varied from one-half hour to 30 hours. Occasionally inexplicable variations occurred in the different classes of milks, but when these were smoothed out by applying a "moving average," a curve was obtained which closely approximates those obtained by the other methods.

CONCLUSIONS

When the bacterial content of a milk was low (less than a million) all of the methods used gave satisfactory results except the direct microscopic count.

The little plates furnished results in one-eighth to one-fifth of the time required by the other culture methods.

The bacterial content of highly contaminated milks could be quickly and satisfactorily determined by either the direct microscopic method, except in case of pasteurized milk, or the reduction test.

The character of the bacterial flora was revealed in a striking way by both the little plate and the direct microscopic count.

ACQUIRED IMMUNITY TO AN ANIMAL PARASITE

F. H. REULING

*From the U. S. Biological Station, Fairport, Iowa, and the Pathological Laboratory,
Northwestern University Medical School, Chicago*

During the summer of 1917, while engaged in the experimental propagation of mussels, the attention of the author was directed to an acquired immunity in fish, acting as the host, to glochidia, the larval form of the fresh-water mussels, acting as the parasite. It was noticed that after two successive optimum infections of glochidia on the gills of a fish (this constitutes about 2,000 per individual with the species used) that the fish, which previously had carried the larval mussel through to maturity, did not permit the complete metamorphosis of a third or any subsequent infection. In every case the glochidia would attach normally, both as to time of attachment and number, but if the fish had had two previous infections or preferably three, the glochidia would drop off in 24-72 hours without any noticeable progress in their metamorphosis. The glochidia during the summer are normally parasitic for two or three weeks during which time their metamorphosis is completed.¹

This artificial immunity to the metamorphosis of an animal parasite was strikingly apparent during the summer in 1917, in the short and long-nosed gar, *Lepisosteus osseus*, and *Lepisosteus platostomus*, which were being used as the specific host for the yellow sand shell, *Lampsilis anodontoides*. Because of the strong vitality of the gar, lots of 8 or 10 were used repeatedly for artificial infections. But with striking uniformity the metamorphosis was not completed on the third or subsequent infections.

The probability of an acquired immunity has been supported by Mr. Thaddeus Surber. Howard² calls attention to Surber's views and mentions that Surber observed sunfish "which received glochidia on the first infection, but not the second." Mr. Austin F. Shira has told me of cases he has observed where an acquired immunity has been produced after two or three infections.

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¹ For a discussion of the length of the parasitic period taking up Schierholz and Harms work see: Lefevre and Curtis, Bull. U. S. Bureau of Fisheries, Document 756.

² U. S. Bureau of Fisheries, Doc. 801, 1914, p. 37.

In speaking of natural immunity, for example, such as the large-mouth black bass, *Micropterus salmoides*, has for the yellow sand shell, *Lampsilis anodontoides*, Lefevre and Curtis³ mention such mechanical factors as configuration of mouth parts, texture of gills, smallness of gill openings, and rapidity of fin movements. Howard in discussing the same condition very aptly says that "it seems not improbable that the tissues or blood of the nonhost possess reactions in the nature of antibodies, precipitins, and other immunizing agents, such as those discovered in the higher vertebrates, while the glochidium is especially adapted to the reactions of the appropriate host."

During the summer of 1918, while still engaged in the propagation of mussels, I planned on determining the more important facts in regard to the acquired immunity encountered during the preceding summer.

Unfortunately, the yellow sandshell, *Lampsilis anodontoides*, which is parasitic⁴ on the gar, did not spawn until fall. Hence other species of host as well as parasite had to be used. Through the ever ready efforts of Mr. H. L. Canfield, I was able to secure an unlimited supply of the Lake Pepin mucket, *Lampsilis luteola*, and a limited number of large-mouth black bass, *Micropterus salmoides*. The fish were separated into lots of 4 or more each and placed in sheltered troughs 12 feet long, 1 foot wide and 10 inches deep, supplied with running water from the Mississippi. The bottom of the troughs was covered with sand and gravel. The fish were fed twice a week with minnows, crayfish, or grasshoppers. The fish remained in excellent condition throughout the entire summer, their vitality increasing rather than diminishing.

To simplify the report, I will give in detail the infections, species of mussels used, and the results obtained from one lot of fish.

The fish to be described are designated in my records as Lot 2-B, composed of 8 adult bass, *Micropterus salmoides*, varying in size from 7-14 inches long and all in excellent condition. Some of these were caught in the Mississippi, others were raised in the ponds at the Fairport Biological Station.

June 23, 1918, these fish were put in one of the troughs already described. On June 25, they were infected with *L. luteola*. The glochidia attached rapidly and it was estimated that about 2,000 had encysted on the gills of each fish. Specimens of the gills were taken every 2nd or 3rd day. The glochidia were well encysted and the metamorphosis proceeded normally. The mussels dropped off after a parasitic period of 18 days. On September 1, well-developed mussels averaging 15 mm. in length were recovered from this infection.

After a 6-day rest the fish were moved to another trough and reinfected with *L. luteola* on July 18. The glochidia attached well and completed their metamorphosis in 11 days, the fish being free of the infection on July 30. On September 5, well-developed mussels averaging 10 mm. in length were recovered from this second infection.

³ Bull. U. S. Bureau of Fisheries, 1912, 30, p. 109.

⁴ For a discussion of the restricted parasitism among the fresh-water mussels, see Surber, T. 1913. Notes on the natural host of fresh-water mussels, Bull. U. S. Bureau of Fisheries, 1913, 32, p. 101.

On August 2, after a 3-day interval, the fish were again moved to another trough and reinfected with *L. luteola*. A control infection, using 4 adult bass which had been raised at the station and had never been infected with glochidia, was made at the same time. On all control infections I have suspended the glochidia from 3 or 4 mussels in a bucket of water and have divided this mixture between the control and experimental fish, thus eliminating the possibility of applying "unripe" glochidia, or glochidia which were unusually active from one mussel to one set of fish and not to the other. In this infection both the control and the original fish (Lot 2-B) received the glochidia rapidly and well, to the extent of about 2,000 glochidia per fish. On examination after 24 hours the gills of the fish which had previously been infected twice showed marked necrosis and sloughing of the epithelial cyst around each glochidium. Under the microscope the glochidia had about them an unusually heavy cyst which, in certain instances, was in the process of being sloughed off along with the glochidia; the glochidia themselves showed a certain amount of disintegration. The shell was still intact and in fact remained so throughout, but between the valves there was considerable cellular debris which had broken off from the glochidium itself. The control fish, examined at the same time, showed well-encysted, normal glochidia. After 48 hours the original lot of fish, except one individual which was the smallest fish of the lot and which will be discussed later, was entirely free from its infection and the gills were a normal, healthy red. The control fish held their infection throughout; the glochidia completed their metamorphosis normally and dropped off in 12 days. Well-developed, growing mussels averaging 5 mm. in length were recovered on Sept. 3 from this control infection.

This result was in accord with my previous observations that the immunity was acquired after two infections and that the glochidia would react the same way on any subsequent infection.

The question now arose whether fish which were the host for more than one species of mussel and had become immune to one of those species would likewise be immune to the other species. It may be stated here that we recognize the large-mouth black bass, *Micropoterus salmoides*, as the host for the glochidia of 4 mussels: *Lampsilis luteola*, *L. ventricosa*, *L. ligamentina*, and *Quadrula plicata*.

To solve this problem I used the 8 bass (Lot 2-B) which had become immune to *Lampsilis luteola*. These fish were allowed to rest 10 days after killing and sloughing off the third infection of *L. luteola*. They were then infected on August 14, with *L. ventricosa*. A control infection was made with 5 previously uninfected bass from the ponds. Both the original 8 fish (Lot 2-B) and the 5 control fish took the infection well. In 36 hours the fish which were previously immune to *L. luteola* had sloughed the infection of *L. ventricosa* entirely off, with the same pathologic changes previously described. The control fish held the infection normally and carried the glochidia to complete metamorphosis in 12 days.

These 8 immune fish were then allowed to rest a week. Their condition was even better than when they were received in June. On August 21, they were infected with *Lampsilis ligamentina*. A control infection was made with three bass which had never carried glochidia. The glochidia attached rapidly and exceptionally well to both lots of fish. The immune fish sloughed off the glochidia just as they had done before. Considerable necrosis occurred in 24 hours and the gills were absolutely clean in 48 hours. The control fish held the glochidia to maturity and dropped them with metamorphosis complete in 17 days.

Unfortunately, it was impossible to obtain any gravid *Quadrula plicata*; hence no infection could be made with this species.

The foregoing account of the immunity induced in the 8 bass described, is typical of the immunity induced in all fish with which I have worked and which have had two or three maximum infections of glochidia during the same summer.

THE NATURE OF THE IMMUNITY

With these facts as presented it became necessary to determine, if possible, the factors which made a nonhost of a fish which had successfully carried at least two infections.

The possibility of a large increase of fibrous scar tissue from the repeated irritation, thus producing a mechanical immunity, had to be considered. Even a superficial consideration makes this seem unlikely, since an increase in fibrous tissue sufficient to interfere with the nourishment of the glochidia would certainly interfere with the respiration of the fish, and, as has been above stated, the fish were in excellent condition throughout. However, a number of sections of the gill filaments from the immune bass and from normal, uninfected bass were made and stained for fibrous tissue. These were examined carefully but in no instance was there any noticeable increase in the amount of fibrous tissue in the immune gills.

Another factor which presented itself was a possible increase in the number of mucous cells around the tip and periphery of each gill filament in the immune bass. The mucous cells on the gills are considered to have two functions: (1) That their secretion is toxic for bacteria and that they thus keep the delicate gills free of infection, and (2) that the mucous secreted washes away dirt and debris which may collect on the gills. Hence, it is conceivable that an enormous

increase in the number of mucous cells, and thus a greatly increased secretion, might make the gills untenable for glochidia. To test this possibility, filaments of the same size were cut from normal and immune bass of the same age. These were sectioned serially, stained for mucous cells, and the cells counted on both immune and normal filaments. Although the number varied considerably there was no constant increase or decrease on either the normal or immune filaments.

With these two possibilities eliminated it was thought probable that the blood of the immune fish had acquired some specific antibody for the glochidia. With this in view, experiments involving two blood reactions were determined on. The first of these was to observe the reaction of the glochidia in the immune serum. To accomplish this the blood of one of the immune bass and the blood of a bass which had never been infected were drawn into sterile test tubes and allowed to remain in the icebox 12 hours. The glochidia of *Lampsilis luteola* were then carefully removed from the marsupium with a sterile pipet and washed 4 times with distilled water. A drop of the immune and normal serum was then drawn from the clotted bloods, placed on cover glasses, and 6-8 gaping glochidia introduced into each. These were inverted over hollow ground slides and sealed with vaselin. In both cases the glochidia immediately snapped shut. The glochidia in the immune and control serums remained the same for about 2 hours. Then the glochidia of the immune serum began to show a striking reaction. The cells of the mantle layer and around the adductor muscle in the glochidia were slowly desquamated and were eventually broken up into cellular debris. In some cases the valves opened partially and the debris protruded to the outside. In other cases the valves remained in fairly close proximity to one another and the debris collected in irregular clumps along the inside margin of the valves. This proceeded until the entire internal structure of the glochidium was destroyed; no further reaction occurred and the valves remained intact throughout. The control remained alive and normal for 48 hours. In other words, the blood of the immune bass contained a cytotoxin for the cells of the glochidia. It will be noticed that this reaction is in accord with the observation I had previously made of the way the glochidia reacted on the gills of the immune fish. The reaction was naturally slower on the gills because the glochidia did not come in contact with as much of the fish's blood at one time. It may be noted here that the glochidia actually do come in contact

with some of the fish's blood and lymph when they first attach. To quote Lefevre and Curtis:³ "Since the hookless glochidia, which are essentially gill parasites and, when taken into the mouth of the fish lodge among the gill filaments, produce abrasions of the delicate epithelium covering the latter, a more or less extensive hemorrhage from the blood capillaries occurs, as may be readily seen from a microscopic examination. It is therefore evident that blood exuding from the gill filaments in the immediate neighborhood of the glochidia must have the same effect as in our experiments, and by exciting vigorous contractions of the adductor muscle furnish an efficient stimulus in bringing about a firm and permanent attachment to the filaments."

This hanging drop experiment has been repeated several times with the glochidia of *L. luteola*, *L. ventricosa*, and *L. ligamentina*. The results have been uniform except that some of the antisera apparently contained a less active cytolysin than others, some requiring 6-12 hours to produce the results.

Since a few of the glochidia must die and be partially absorbed by the fish's blood and since all of them come in fairly close contact with the blood, it was thought possible that a precipitin was formed in the blood of the immune bass.

Uhlenhuth⁵ after 24 days produced a precipitin in a rabbit fed on egg white and Metalnikoff⁶ produced a hemolysin in the serum of rats fed on horse blood, hence it was deemed not unlikely that a precipitin would be formed in the fish from such repeated and close contact of the glochidia with the respiratory apparatus.

The amount of blood obtainable from a bass is very limited, hence capillary serologic tubes were used for this work. The blood was collected in sterile test tubes and put in an icebox for 12 hours. The blood from normal, uninfected bass was used as a control. Glochidia were removed from the marsupium of a gravid *L. luteola*, ground to a homogeneous mixture in a sterile mortar with normal salt solution, and centrifuged. The glochidia extract was used in dilutions of 1:1, 1:5, 1:20, 1:50, 1:100, and 1:200. One drop of the immune serum was added to each dilution. Controls of normal serum were run side by side with the serum being tested. The technic used by Nuttall⁷ was followed fairly closely. The results were not striking enough to

³ Ztschr. f. Hyg. u. Infektionskr., 1897, 26, p. 384.

⁶ Centralbl. f. Bakteriolog., 1901, 29, p. 531.

⁷ Blood Immunity and Blood Relationship, 1904.

invite much confidence. The 1:5 dilution of the glochidia extract with one drop of the immune serum added to it showed a fairly constant cloudiness and a light flocculent precipitate after 24 hours at room temperature. The control remained clear throughout. This was the only dilution in the several sets of tests which showed a constant precipitate—and in one set even this dilution failed. Other dilutions would often show a cloudiness or opacity, but, as Nuttall points out, no conclusions can be drawn from such cloudiness. This is especially true when working with a substance such as the glochidial extract. Because of the shortness of time more extensive tests were not undertaken. Hence I do not care to draw any final conclusions as to the presence or absence of a precipitin. In general, the tests indicated that a very weak precipitin was present in the anti-serums, but it will require more extensive work to determine this point.

THE FACTORS INVOLVED IN PRODUCING THE IMMUNITY

Several factors might be involved in producing the immunity. If we assume, with Lefevre and Curtis,³ that the epithelial cyst formed about the glochidium is stimulated by a secretion of the glochidium as well as by the mechanical "bite," and when we further consider that these stimuli are applied to the extent of about 2,000 per fish at a single infection, it does not seem improbable that antibodies to this stimulus and to the glochidia might be formed similar to the antivenin which Calmette⁸ has produced for snake toxins, the hemolysins Metalnikoff⁶ produced, or the antibodies including the precipitin which Bashford⁹ has produced in a rabbit treated with croton. There exists also the possibility that the antibodies, especially the cytolyisin, are stimulated to production by the absorption of glochidia which have died on the fish during their metamorphosis. From microscopic examination there is fairly good evidence that a few of the glochidia do die on the gills. When the fish carry nearly a maximum infection, as they do in the artificial propagation work, the number which die, although very limited, must proportionately increase. Hence, if these are absorbed while still in the cyst we have a fairly plausible explanation of the cause of the immunity. For, as Schültze¹⁰ produced an antiserum for the vegetable protein "Roborat," for muscle albumen, and a generalized yeast precipitin for the yeasts,

⁸ Ann. de l'Inst. Pasteur, 1895, 9, p. 225.

⁹ Jour. Pathol. and Bacteriol., 1902, 8, p. 59.

¹⁰ Deutsch. med. Wchnschr., 1902, 28, p. 804.

it seems likely that antibodies would be formed by the absorption of albumen of the glochidia.

Late in the summer of 1918 in an effort to induce a similar immunity in a normal bass I made 3 injections into the abdominal cavity of an adult bass of filtered, ground glochidia. The injections were made 4 days apart and consisted of 0.5 c c, 1 c c, and 2 c c. When this fish was infected along with a known immune and a known normal fish it held the glochidia equally as well as the normal fish, while the immune shed the infection within 36 hours as previously described. In other words, the immunity was not induced by the injections made. The failure of this may be explained in one of two ways: (1) That the injections were small and did not last over a long enough period of time, and (2) that the glochidia were filtered. It may be that the shells of the glochidia are involved in the immunity production or that the immunity producing substance was filtered out. It will be recalled that Graham-Smith¹¹ found that *Limulus* serum unlike mammalian and avian serums, when passed through a porcelain filter no longer produced a precipitin when tested with anti-*Limulus* serum, while the unfiltered serum did.

The question is now naturally raised: How long will the immunity persist and what effect will it have on the commercial application of the artificial propagation of the fresh-water mussel?

These two questions are unanswered as yet, and only the surveillance of the immune fish from year to year will determine it. If the immunity does last for several years it will constitute an additional hazard in the artificial propagation of mussels. This is true because under the methods now in use the fish are seined from the river, infected in an hour or so, and turned back again. There is at present no way of telling whether these fish hold the infection or not. If with the extension of artificial propagation a great many fish become immune in a restricted area, the fish may be infected over and over again with the false idea that they are retaining the infection each time, while in reality they may be sloughing them off in a day or two. This suggestion is merely offered as one of the factors which must be considered in the event of more intensive artificial propagation work and applies especially to any attempt at trough, pond, or small lake propagation on a commercial or even practical basis.

¹¹ Blood relationship amongst the lower vertebrates and arthropoda, etc., as indicated by 2500 tests with precipitating antisera, 1904.

The past summer there was under consideration one bass of normal size which from the first of the season refused to hold the glochidia. I tried to infect this fish repeatedly, but it would invariably slough them off in 24-48 hours. This bass is one that remained in the ponds of the station over winter, but there is no record to show how many times it was infected during the preceding summer. It acted throughout like those fish which had the artificial immunity induced in them as previously described, and it would seem likely that this fish had acquired an immunity during the preceding summer and still maintained it over the period of a year.

Early in the paper the fact was mentioned that one of the small bass—apparently a 2- or 3-year old belonging to the described Lot 2-B—did not become immune after the second infection but required a third infection before it exhibited the same reactions which the larger and older fish showed after 2 infections. In my records this seems to be a fairly constant condition. I would explain this circumstance by pointing out that the older bass have had a number of natural and hence small infections, and that thus they have been started toward an acquired immunity although they might never have reached complete immunity under natural conditions. But when these fish are artificially infected with 2,000 or more glochidia their immunity is completed very rapidly—in my records some of the very large bass have become immune after one infection. On the other hand, the small bass will often require 3 infections. This is natural since the younger bass have not received any preliminary glochidia and hence require 3 heavy infections to produce the same degree of immunity.

Prof. H. S. Davis has called my attention to certain unreported conditions produced by myxosporidia. On young buffalo fish, *Ictiobus bubalus* and *I. cyprinella*, in ponds where gill species of the myxosporidia were abundant, he has found practically a 100% infection, while on large and old buffalo fish, even in the same pond, the infection will be very light or totally absent. Since these myxosporidia encyst themselves and eventually come in contact with the blood stream it is possible that the older fish have acquired an immunity to the parasite similar to the one described above.

Furthermore, Wilson¹² calls attention to the fact that the presence of glochidia and copepods are antagonistic to one another. That is,

¹² Bull. U. S. Bureau of Fisheries, 1914, 34, p. 333.

if a fish has a heavy infection of copepods it will receive only a limited number of glochidia and vice versa. It may be stated here that the experimental fish which have been described in the present report have been exceptionally free from copepods. He goes on to say that this incompatibility is probably chemical or physiologic in its action. The same author¹³ introduced free-swimming copepodid larvae, gills of a fish heavily infected with glochidia, and gills free of glochidia into an aquarium. On the following morning the gills that had no glochidia were well covered with copepodid larvae. But none of these larvae had attached to the gills that were already occupied by glochidia. This may point to a delicate secretion which the glochidia have, which is instrumental in producing the immunity and at the same time is antagonistic for other forms such as the copepods.

CONCLUSIONS

An immunity to the metamorphosis of glochidia is produced in fish after repeated heavy infections.

The fish becomes immune to all the species of glochidia for which it is a host.

The immunity is a blood immunity and is not concerned with mechanical factors.

¹³ Bull. U. S. Bureau of Fisheries, Doc. 854, 1917, 35.

THE SPECIFICITY OF BACTERIAL PROTEOLYTIC ENZYMES AND THEIR FORMATION

HAROLD S. DIEHL

*From the Department of Bacteriology and Immunology, University of Minnesota,
Minneapolis**

Ever since the recognition of enzymes as the active agents in a large number of vital processes of both multicellular and unicellular organisms, much work has been done to determine their nature and properties. Through this we have learned much about them, but a great deal is still left unknown, mainly because it has been impossible to obtain them in a pure form. So at present we can study them only indirectly, that is, by investigating their properties and their modes of action.

One of the first properties assigned to amylases, lipases, and proteases was that of specificity. Even among the enzymes acting on the several carbohydrates a certain amount of specificity was observed; but the nature of this specificity could not be determined because it appeared that these ferments were inseparable from the bacterial cells. They are what we now know as endo-enzymes of bacteria. With the proteolytic enzymes, however, more work was possible because these are given off by the cells into the surrounding medium, and are therefore susceptible of isolation.

The action of these enzymes on the proteins offered a good opportunity for the study of specificity because of the great differences between the several types of proteins. If enzymes really be specific, then one would expect that no single enzyme would be able to act on all of the widely different types of proteins. On the other hand, it hardly seems probable that a different enzyme exists for each. For, if this were actually the case, digestive enzymes such as pepsin and trypsin would have to be considered not as units, but as combinations or mixtures of many distinct ferments. Such a conception does not seem probable, nor is it borne out by the experiments of Fermi¹ and others, who came to the conclusion that the proteolytic enzymes of the digestive juices are units and are not specific for individual proteins.

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Fermi,¹ in discussing this question, quotes the views of Fischer and Abderhalden. Their opinions are very similar and well worth repeating here. Emil Fischer states: "That most of our common ferments are mixed is very probable. But how far one should go in the differentiation of them is hard to say without experiment; and I must admit that most experiments which have been carried out in this connection possess but very little conclusive force. In any case, I believe that it is an exaggeration to assume for each protein that is attacked by pepsin and trypsin a special enzyme; for the necessary conclusion would be that one must assume for each glucosid a special enzyme, namely, for beta-methyl-glucosid and for beta-phenyl-glucosid. According to chemical analogies, I must hold this as being very improbable." Abderhalden expresses very much the same view when he says: "It is quite probable that pepsin and trypsin are not units, still, any sure proof of a gelatinase, casease, etc., is lacking in my conviction at the present time. So long as one is forced to work with unknown substrata and unknown ferments, sure conclusions are very hard to get."

In investigating this problem numerous proteins have been used to test enzyme action but gelatin has been more popular than any other. This is so chiefly because gelatin is easily prepared and any proteolytic action on it is discernible by direct observation. For this reason the digestion of gelatin has come to be looked on by many bacteriologists as an index of the proteolytic activity of bacteria, and in most cases it is quite satisfactory. Next to gelatin, casein probably has been most used, with variations of technic to determine the amount of its digestion. Hemoglobin, fibrin and other proteins have been tried with more or less satisfactory results.

REVIEW OF THE LITERATURE

Pollak,² after much experimenting, was convinced, (1) that the gelatinases are not identical with trypsin and (2) that a specific gelatinase exists. He bases this latter conclusion on the fact that in immune serum he could demonstrate an antigelatinase, which would suspend the gelatinolytic activity of solutions but would leave uninfluenced the serolytic. Hattori³ also claims to have demonstrated a specific gelatinase by the use of chemicals which would destroy the albumino-, sero- and fibrino-lytic powers of a solution but would leave unimpaired the gelatinolytic power.

On the other hand, Duclaux⁴ thinks that the gelatinases and caseases are identical, and also assumes the identity of both of these with trypsin. And

¹ *Centralbl. f. Bakteriol.*, 1, O., 1913, 68, p. 433.

² Cited by Marras (*Hofmeister's Beitr.*, 1905), 6, p. 95.

³ Cited by Marras, *Arch. internationale de pharmacol.*, 1908, 18, p. 255.

⁴ *Microbiologie*, 2, p. 618.

Fermi,⁵ after extensive work with ferments obtained from animals and plants, including bacteria, concludes that the proteolytic enzymes are identical with trypsin. He found that, when fibrinases and caseases were present, gelatinases were also present; but that some bacteria which produce gelatinases do not produce fibrino- and caseino-lytic enzymes. From these findings he draws the conclusion that the same enzyme acts on all proteins, but that different proteins show various degrees of resistance.

De Waele and Vandeveld⁶ also believe that the differences in bacterial proteolysis are differences of intensity only. They state that on the different nitrogenous mediums all bacteria act quite similarly and that all organisms which liquefy gelatin also vigorously attack casein. To them this parallelism indicates that the liquefaction of gelatin and the solution of casein are due to one and the same ferment. However, they also find that bacteria produce various amounts of proteolytic ferments and that these ferments differ somewhat in their action; but that these differences depend not so much on the bacteria as on the conditions and environment under which the enzymes are produced.

By the use of blood agar Eijkmann⁷ demonstrated an enzyme liberated by *B. diphtheriae* which was hemolytic but not proteolytic; and one liberated by a coccus from the air which digested gelatin but did not affect hemoglobin. To account for these differences he suggests several possibilities: (1) That different kinds of proteolytic enzymes exist, one of which will most readily act on blood and another on gelatin and (2) that some metabolic products of the bacteria other than the ferments are responsible for some of the actions. The latter possibility is one which has not been mentioned by any other workers and is decidedly the less probable of the two. He also utilized agar-elastin plates and on these observed the action of an enzyme that seemed to him identical with "the peptonizing ferment which liquefies gelatin." But, he states that there was always some incongruity in the clearing of the elastin and the liquefaction of the gelatin. This is exactly what one should expect, since gelatin and elastin though very closely related chemically are nevertheless not identical.

Mavrojanis,⁸ working with 8 types of bacteria, found that 5 of these digested gelatin to the stage of gelatoses; while the other 3 would carry them through to gelato-peptones. The gelatoses can be resolidified by means of formol while the gelato-peptones are unaffected by it. From these results he concluded that at least two kinds of ferments were responsible for the breaking down of gelatin, and that each of these was specific. These results, however, were soon refuted by Bogoluboff⁹ and Tiraboschi;¹⁰ but the work remains of some value because it helped to stimulate other investigations along the same lines. The latter authors found that the production of gelatoses or of gelato-peptones depends not so much on the type of ferment as on the temperature, the reaction of the medium, and the length of time which the ferments are allowed to act.

⁵ Centralbl. f. Bakteriöl., 1913, 1, O., 68, p. 433; 1913, 69, p. 465.

⁶ Centralbl. f. Bakteriöl., 1, O., 1905, 39, p. 353.

⁷ Centralbl. f. Bakteriöl., I, 1901, 1, p. 841.

⁸ Compt. rend. Soc. de biol., 1903, 55, p. 1605.

⁹ Bull. de l'Institut. Pasteur, 1907, 5, p. 429.

¹⁰ Bull. de l'Institut. Pasteur, 1905, 3, p. 922.

The review of the literature shows a great diversity of results and conclusions, but gives the general impression that proteolytic enzymes are probably not specific. Still such a conception leaves unexplained some of the phenomena which these enzymes often exhibit. The statement is frequently encountered that the conditions and environment greatly influence the formation and properties of bacterial enzymes. Of all the factors that are included under the conditions and environment of enzyme formation, the composition of the medium is probably the most important.

In this connection the question suggests itself as to whether enzymes are preformed in the bacterial cell or whether they are produced by the organism only when the need for them arises. From our knowledge of vital processes in general it seems more probable that the bacterial cell should be endowed with the ability to elaborate ferments than that it should be a storehouse for the many ferments of which it has need at one time or another; or even that it should form all the ferments within its power each time that there is need for any one of them. The work of Jones¹¹ substantiates this view by showing that *B. proteus* will not form gelatinases on gelatin when any glucose is present; but that as soon as the glucose has been used up the gelatinases appear. This would indicate that the ferments are not preformed in the bacterial cell, but that they are elaborated in response to need. Such a concept would further suggest that the many different kinds of nitrogenous mediums would probably stimulate the production of more than one type of enzyme. For example, it does not seem probable that an amino-acid and a highly complex protein would call forth the production of identical ferments. The present investigation was undertaken to determine, if possible, the extent of the influence of mediums on ferment production.

TECHNIC

The cultures to be studied for proteolytic enzymes were incubated at 37 C. for a period of 8 days. At the end of this time they were filtered through a sterile Berkefeld filter and 0.5 c.c. of the filtrate was added to sterile gelatin and sterile casein solution. Control tubes were made in each case for comparison; and tests as to the sterility always made, all filtrates showing contamination being discarded.

The gelatin mediums used to test for proteolysis contained meat-infusion broth and 15% of gelatin. To several tubes of this in the hardened form was added a portion of each filtrate and the tubes were placed in an incubator at 20 C. Several other tubes of gelatin were liquefied before the

¹¹ Jour. Infect. Dis., 1916, 19, p. 33.

filtrate was added and then placed in an incubator at 37 C. Liquefaction in the latter case was determined by placing the tubes in ice water until the controls hardened. The results obtained by both methods coincided in every case, although the action was more rapid at the higher temperature.

The casein solution was made up according to the method of Föld and Gross.¹² Ten gm. of Merck casein were dissolved in 100 cc of 10% Na₂CO₃ solution, enough 10% HCl added to just neutralize it and the volume made up to 500 cc with physiologic salt solution. The whole was then filtered, and diluted with physiologic salt solution until the medium showed a definite clouding on the addition of acetic acid, but no heavy precipitate. This was placed in small test tubes, 5 cc to a tube, and sterilized. To test for enzymes 0.5 cc of the filtrate under examination was added to each of several tubes and incubated for 24 hours, after which undigested casein was determined by the addition of 0.2 cc of a solution of 45 parts of 90% alcohol, 5 parts of glacial acetic acid, and 50 parts of distilled water. The absence of any precipitate indicated that all the casein had been digested. With each tube of casein a control was run for comparison.

Various methods have been utilized for the detection of gelatinases in bacterial cultures. Of these, probably the most widely used is the carbol-gelatin method in which 0.5 cc of the culture to be tested is added to a tube of gelatin containing 5% carbolic acid. This is supposed to prevent the growth of the bacteria but to have no influence on the action of the enzymes. Eijkmann¹³ separates the enzymes from the bacteria by dialysis through agar, but the method seems impracticable when carrying out a large number of tests. Another method widely used is the one which gave the best results in our hands, namely, the separation of the enzymes from the bacteria by the filtration of the culture through a sterile Berkefeld filter. The method is laborious but, when properly carried out, very satisfactory.

Concerning the time at which the proteolytic activity of a culture is at its maximum, different views can be found. Von Gröer¹⁴ states that the most gelatinases of *B. prodigiosus* are found in a culture 3 weeks old. Malfitano and Strada¹⁵ found that on agar slants the proteases increase up to 24 hours and then decline. Mesernitsky¹⁶ agrees with the work of Von Gröer on *B. prodigiosus* in finding that the gelatinases appear at the beginning of the 6th day and increase up to the 21st day. Bertiau¹⁷ working with *B. pyocyaneus* and *B. subtilis* concluded that notable ferments exist in broth cultures at the end of 2 days and increase up to the 8th day, after which they decrease slowly. In our work all cultures were tested at the end of 8 days and, in case of negative results, were tested again after a greater length of time, but never did positive results appear in cultures which were negative at the 8th day.

EXPERIMENTS AND RESULTS

The content of the mediums on which the organisms were grown was varied in order to determine what effect this would have on enzyme production. Gelatin itself was first tried and proteolytic

¹² Cited by Marras (Centralbl. f. Bakteriöl., 1, O., 1914, 74, p. 505).

¹³ Centralbl. f. Bakteriöl., 1, O., 1904, 35, p. 1.

¹⁴ Biochem. Ztschr., 1912, 38, p. 252.

¹⁵ Compt. rend. Soc. de biol., 1905, 59, pp. 120, 195 and 197.

¹⁶ Biochem. Ztschr., 1910, 29, p. 104.

¹⁷ Centralbl. f. Bakteriöl., 1, O., 1914, 74, p. 374.

enzymes were found to be produced by each of the organisms used. These enzymes, however, were not specific gelatinases because, although they liquefied gelatin rapidly, they also promptly digested the casein. This agrees with the findings of De Waele and Vandeveld⁶ and others, and is the basis for some of the conclusions as to the identity of gelatinases and caseases.

When the bacteria were grown on the casein solution, proteases were again obtained which attacked both gelatin and casein and, with the qualitative methods employed, no difference between the activity of these ferments and the ones obtained from the gelatin mediums was apparent. However, later experiments lead us to conclude that with more accurate methods for the quantitative estimation of proteolysis some differences will very probably be found. The filtrates from cultures on meat-extract and meat-infusion both likewise were found to contain ferments which would rapidly digest both gelatin and casein.

After obtaining apparently identical ferments from these several mediums, all of which contained numerous complex proteins, the other extreme was resorted to, that is, a medium was utilized which contained no organic nitrogen. It is one which Magoon¹⁸ recommends for the cultivation of tubercle bacilli and on which all of the common bacteria will usually grow well. It is made up as follows:

Ammonium phosphate, dibasic.....	1.75 gm.
Potassium phosphate, dibasic.....	0.25 gm.
Sodium phosphate, dibasic.....	0.50 gm.
Magnesium sulphate	0.50 gm.
Glycerol	20.00 c c.
Distilled water qs. ad.....	1,000.00 c c.

When all of the salts are dissolved, this is sterilized by fractional sterilization, after which it may be kept indefinitely and used as desired. Although this medium, due to the presence of the glycerin, is not strictly inorganic it will be called an "inorganic medium" in this paper for the sake of brevity.

When inoculated into this medium, which had been placed in small flasks, 100 c c to a flask, the organisms all developed rapidly and within a few days a heavy growth was obtained. At the end of 8 days a portion from each flask was filtered and the sterile filtrate added to gelatin and to casein solution as described. But on incuba-

¹⁸ Washington Agricultural Experiment Station, Bull. 132.

tion no digestion occurred in any of the tubes. Other portions of the cultures were filtered and tested at 14 and 21 days, but in no case were any enzymes obtained which would attack either the gelatin or the casein.

By means of the next experiments it was hoped to determine how close to gelatin and casein in chemical composition the mediums would have to be to stimulate the production of gelatinases and caseases. With this object in view peptone was added to the above described medium in a quantity sufficient to make a 1% solution. On this medium the organisms all grew vigorously, and it was found that they produced enzymes which would digest both gelatin and casein.

This showed the necessity for utilizing some substances still more simple as sources of organic nitrogen and, as asparagin is frequently used in nonprotein mediums, it was next tried, enough of it being added to the original medium to make a 1% solution. On this the bacteria showed rapid development and in the filtrates from the cultures enzymes were found to be present which both liquefied the gelatin and digested the casein, and with apparently equal rapidity.

These results led us to study the literature on the chemical composition of gelatin and casein by Fischer, Abderhalden and others. According to Matthews,¹⁹ in addition to various percentages of most of the amino-acids, both of the above proteins contain aspartic acid, gelatin 0.56% and casein 1.4%. Gelatin also contains 16.5% glycocoll and casein none, while casein contains 4.5% tyrosin and 1.5% tryptophan and gelatin contains neither. Of these amino-acids tryptophan was not available, but glycocoll and tyrosin were utilized for our experiments, 1% solutions of each being made up with the "inorganic medium" as a base.

In the mediums containing glycocoll each of the 3 organisms were allowed to develop as in previous experiments but growth was found to be more slow. However, after several days a vigorous growth appeared in each of the flasks, and at the end of 8 days the cultures were filtered and portions of the filtrates tested for enzymes in the manner already described. Each one of these filtrates from the mediums containing glycocoll showed marked liquefaction of gelatin, but had no action on the casein. The rate of the liquefaction of the gelatin was also marked, being noticeably more rapid than that caused by the filtrates from any of the mediums previously used, even from

¹⁹ Physiological Chemistry, 1916.

gelatin itself. But on the casein solution there was no action, even when allowed to remain in the incubator for several days. Other samples from these cultures were filtered and tested at various intervals up to 3 weeks, but the results obtained were always the same.

On the mediums containing the 1% of tyrosin growth was more rapid but the filtrates less active. In the tubes of gelatin, which were inoculated with filtrates from this medium, there was absolutely no evidence of liquefaction, either at 20 C. or at 37 C.; but in the casein solutions there was quite marked digestion by the filtrates from the cultures of each of the organisms. This digestion, altho not so rapid as that caused by trypsin, was apparently of about the same intensity as that obtained with the filtrates from broth, gelatin, peptone and asparagin.

In tabular form these results appear as follows:

TABLE 1
RESULTS OF EXPERIMENTS WITH VARIOUS MEDIUMS TO DETERMINE ENZYME PRODUCTION

Source of Enzymes	Liquefaction of Gelatin	Digestion of Casein
Gelatin:		
B. pyocyaneus	+	+
B. prodigiosus	+	+
B. subtilis	+	+
Broth:		
B. pyocyaneus	+	+
B. prodigiosus	+	+
B. subtilis	+	+
Agar slant:		
B. pyocyaneus	+	+
B. prodigiosus	+	+
B. subtilis	+	+
"Inorganic mediums":		
B. pyocyaneus	0	0
B. prodigiosus	0	0
B. subtilis	0	0
1% peptone:		
B. pyocyaneus	+	+
B. prodigiosus	+	+
B. subtilis	+	+
1% asparagin:		
B. pyocyaneus	+	+
B. prodigiosus	+	+
B. subtilis	+	+
1% glyccoll:		
B. pyocyaneus	+	0
B. prodigiosus	+	0
B. subtilis	+	0
1% tyrosin:		
B. pyocyaneus	0	+
B. prodigiosus	0	+
B. subtilis	0	+

DISCUSSION OF RESULTS

The fact that nonspecific proteases can be obtained from cultures on gelatin, casein and nutrient broth has frequently been mentioned and is of no special significance in this paper, except to show the basis for most of the conclusions as to the identity of proteolytic enzymes.

These conclusions seem perfectly logical in view of such results, and the ones reached by us would have been very similar, had not experiments with other mediums thrown further light on the subject.

The finding of no gelatinases or caseases on mediums containing no organic nitrogen at first appears contradictory to the findings of some other writers, notably Jordan,²⁰ Aubel and Colin,²¹ and Abbott and Gildersleeve.²² But, on close investigation of their work, one finds that the "nonprotein" mediums of which they write always contain organic nitrogen in one form or another. Jordan, for example, in his experiments varies the salt content of the mediums, but in each case asparagin is found to be present. Aubel and Colin use asparagin or ammonium aspartate as the source of nitrogen. With such findings we can well agree, but on mediums containing no organic nitrogen we are forced to conclude that enzymes are not liberated by the bacteria. These results seem significant in the study of enzyme formation and furnish further evidence that bacterial enzymes are not preformed in the cell but are manufactured by the organism as the need for them arises.

The formation of both gelatinases and caseases on the mediums containing 1% of peptone is not surprising and has very little significance because peptone is a substance of rather indefinite chemical composition, which is found as an intermediate step in the breaking down of all proteins. For this reason, if it stimulated the production of any proteases at all, these would be expected to attack one protein as well as any other.

With asparagin as a source of nitrogen the production of gelatinases and caseases at first seems surprising and appears as another argument against the specificity of proteases. It is one which has been mentioned in the literature and would be a strong one were it not for the fact that asparagin is an amin of asparatic acid which is present in both gelatin and casein. Thus, proteolytic enzymes formed on mediums containing asparagin as a source of nitrogen might be expected to attack casein as well as gelatin. In regard to enzyme production in mediums containing inorganic salts plus asparagin, Jordan²⁰ states that the enzymes are produced in just as large amounts as they are in gelatin or in broth, but that a longer time is necessary. Our results, although not quantitative, seem to agree with this.

²⁰ Biol. studies of pupils of W. T. Sedgwick, Boston, 1906, p. 124.

²¹ Compt. rend Soc. de biol., 1913, 74, p. 790.

²² Jour. Med. Research, 1903, 10, p. 42.

As to the nature of this enzyme our work merely shows that it is proteolytic, digesting both gelatin and casein. Effront and Ehrlich,²³ however, inoculated solutions of asparagin with yeast and obtained ammoniacal nitrogen. The filtrates of such cultures also had the property of breaking down asparagin and the amount transformed was proportionate to the time. This signified to them that amidase is formed which is responsible for the action. On the other hand, Walker and Kendall,²⁴ after studying the proteolytic enzymes of *B. proteus*, conclude that the one which liquefies gelatin is in a sense at least a preparatory one, preparing the protein for assimilation and that "deaminization is an independent phenomenon, associated with the intracellular utilization of the products of proteolysis." This work makes it appear as though there must be two enzymes formed on asparagin, but it is the proteolytic enzyme rather than the deaminizing one with which we are interested.

On glyocoll we found growth to be less vigorous than on the other mediums. This agrees with the observations of Bielecki²⁵ who found that bacteria will grow better on asparagin than on any of the other amino-acids which he investigated, but that some development is obtained on all. Franzen and Egger²⁶ also found that of asparagin, alanin, and glyocoll, asparagin forms the best source of nitrogen for *B. prodigiosus*, while the least growth is obtained on glyocoll. The enzymes obtained from this medium were very marked in their action, the gelatin being liquefied with surprising rapidity and the casein remaining unchanged.

On the mediums containing tyrosin the results were just as striking, the gelatin showing absolutely no liquefaction while the casein was progressively digested. The results seem quite significant in view of the fact that gelatin contains glyocoll but no tyrosin, while casein contains tyrosin but no glyocoll.

The utilization in a similar manner of other amino-acids, particularly tryptophan, would doubtless further demonstrate this point but, due to the shortage of chemicals in consequence of the present war, it has been impossible to obtain them for this piece of work. However, the ones which were used point very strongly toward the conclusion that the amino-acids play a very important part in the determination of specificity among proteolytic enzymes.

²³ *Compt. rend. Acad. de sc.*, 1907, 146, p. 779.

²⁴ *Jour. Infect. Dis.*, 1915, 17, p. 442.

²⁵ *Compt. rend Soc. de biol.*, 1911, 70, p. 100.

²⁶ *Centralbl. f. Bakteriol.*, 1, Ref., 1915, 63, p. 49.

It is as yet hard to say just how comprehensive the conclusions should be which follow from such findings; but it appears that, on mediums containing no organic nitrogen, no proteolytic enzymes are formed, and that, on mediums containing but one amino-acid as a source of nitrogen, the enzymes which are formed will attack only proteins which contain that particular amino-acid. In other words, this enzyme seems specific for proteins containing that one amino-acid. Thus, the enzymes formed on most amino-acids tend to show very little specificity because the great majority of amino-acids, like aspartic acid, are present in practically all of the proteins. Also, the ferments, which are formed on mediums containing either many amino-acids or a few more complex nitrogenous compounds, would have the ability to attack large numbers of proteins, thus appearing to be entirely without specificity.

Experiments similar to the ones described in the foregoing are apparently unmentioned in the literature. Consequently the results may offer a new foundation on which to base our ideas as to the specificity of bacterial proteolytic enzymes. That these enzymes are not specific for individual proteins has been the opinion of many reliable workers and yet the conclusion that one and the same enzyme breaks down all proteins does not follow from their work. They also state a priori that it is unreasonable to assume for each protein and protein derivative a special enzyme. The results of our experiments do not contradict this. They indicate rather that the number of ferments which a bacterial cell can liberate is limited and depends on the amino-acids present.

As to whether the amino-acids in the free and in the combined state exert the same influence on enzyme production has not yet been established. The results obtained by growing the bacteria on the usual protein mediums do not lead to definite conclusions concerning this point because one cannot be certain that these proteins do not contain some free amino-acids, particularly after having been exposed to the high temperatures necessary for sterilization. Some experiments at this laboratory to determine this point are as yet unfinished, but it is hoped that they may eventually throw some light on the subject. The method employed is as follows: Sterile gelatin is subjected under sterile conditions to dialysis for a time sufficient to remove from it all crystalloid substances. Then sterile salt solution is added to give the desired salt concentration and it is inoculated with bacteria which have

first been transplanted several times on the mediums containing no organic nitrogen. The object of these transplants is to obviate the possibility of carrying any amino-acids or ferments onto the gelatin along with the bacteria. As can easily be seen, the difficulties in carrying out this technic are great and as yet no satisfactory results have been obtained. However, by a technic such as this we hope to procure some further definite information as to whether pure proteins stimulate the production of enzymes. This would confirm the work of Sperry and Rettger,²⁷ who found that pure egg albumin, serum albumin, and edestin were unattacked by a variety of proteolytic bacteria in the absence of other organic sources of nitrogen.

But, whatever the results of this experiment may prove to be, the conclusions from those already completed will be unaffected, namely, that the amino-acids are important factors in the determination of the nature of the enzymes which are formed, and that these enzymes are probably not specific for the proteins but rather for the amino-acids which go to make them up.

In regard to the enzymes formed on complex proteins several possibilities suggest themselves: (1) that only one enzyme is formed, and that this is modified by the presence of the various amino-acids so as to attack them all, or (2) that a separate enzyme is formed to correspond to each amino-acid present, and that these different enzymes are present approximately in the same proportion as the amino-acids which stimulated their formation. From our work the latter possibility seems the more probable, although none of the results show this conclusively.

With such results as a basis, we may be able to modify somewhat our ideas of the formation and the action of bacterial proteolytic enzymes. First of all, the enzymes are dependent on some inherent property of the organisms themselves. This is evident from the fact that some bacteria do not have the ability to form certain ferments, no matter on what mediums or under what conditions they are cultivated. For example, *B. typhosus* and *B. dysenteriae*, etc., have never been known to form gelatinolytic nor caseinolytic enzymes, and, although *B. coli* produces an enzyme which will form indol from peptone, it liberates none which will attack gelatin.

A second factor, or group of factors, which influences enzyme formation may be called general conditions. This includes the temperature, the reaction of the medium, the time of growth, etc., all of

²⁷ Jour. Biol. Chem., 1915, 20, p. 445.

which have a greater or less effect on the production of enzymes by bacteria, and on which much exhaustive work has been done by such men as Kendall, Von Gröer, Jordan, Abbott and Gildersleeve, Bertiau, Emmerick, Fermi, etc. Their work shows that in the formation of enzymes there is for each organism a maximum, minimum and optimum temperature, and that in most cases the optimum is about 37 C. The optimum chemical reaction is also variable, a very slightly alkaline medium seeming best for the production of most enzymes. However, as they will act nearly as well in a neutral or slightly acid medium, this point seems to be of no great importance. The quantity of enzymes present in cultures of different ages has been discussed earlier in this paper. All of these observations, although of importance in the study of enzymes, show differences of quality only and so are not particularly pertinent to this question, for which reason they will not be discussed in greater detail.

A third and most important factor in determining the character of the enzymes produced is the composition of the medium on which they are formed. This is demonstrated by the experiments reviewed in this paper, the results of which may be put in general terms as follows: If a protein medium is inoculated with an organism which has the ability to form ferments to correspond to a large number of amino-acids, enzymes which will attack practically any protein can be obtained from the culture. This may be due to the fact that, apparently at least, ferments are formed to correspond to all of the amino-acids present, and that these ferments then attack the acids in whatever form they find them. On mediums containing less complex nitrogenous substances or only amino-acids the same bacteria will give off ferments to correspond only to the amino-acids present, and these ferments will attack only substances containing these same acids. The vigor with which they will attack these substances seems to depend on the relative amino-acid content of the mediums on which they are formed and that on which they act, namely, if the medium on which the enzymes are formed contains only asparagin and glycocoll and the medium on which they are inoculated contains only a small percentage of one or the other, the digestive action will be much less marked than it would be if the protein medium contained more of one or both of the same acids. That is, the amino-acid content of the medium determines the specificity of the enzymes formed.

On the basis of these findings it seems that many of the so-called "irregularities of bacterial action" may be explained. Every bacteriologist finds organisms which, although corresponding according to morphology and pathogenicity to known types, show some "irregularity" in their action on culture mediums. Later, on a different lot of mediums or possibly at a different laboratory, the same organism may show the action on culture mediums which was previously expected, and the worker, at a loss for an explanation, concludes that it is merely another example of the "irregularity of bacterial action." Occasionally there may be some truth to this conclusion, but probably very often this "irregularity" would never have appeared if the chemical composition of mediums were always the same.

These same observations of the effect of mediums on bacterial ferments will probably furnish an explanation for many of the "new strains" of bacteria which are described, but are never verified by other workers. They usually differ from well established strains only in some minor points, and slight differences in the chemical composition of the mediums on which they are grown could well account for these.

Another phenomenon for which this conception of ferment activity seems to offer an explanation is the apparent incongruity of the action of some bacteria on mediums. For example, *B. proteus* and *B. subtilis* are well known to be strongly proteolytic and exhibit typical proteolytic actions, such as the liquefaction of gelatin and the formation of indol. On the other hand, *B. coli* and some of the dysentery group, although without action on gelatin, still form indol in large amounts from peptone. These organisms are not considered proteolytic and yet they unquestionably exhibit proteolytic activity when they split off tryptophan from peptone and then break down the tryptophan itself to form indol. If we accept the theory that all proteolytic enzymes are the same, we must expect these organisms which show proteolysis on peptone to liquefy gelatin. On the other hand, if we consider this in view of the findings given in this paper, the action does not appear at all surprising. Here are several organisms which have the inherent ability to form enzymes which will attack only one-amino-acid and that is tryptophan. And in peptone we have a protein derivative which contains tryptophan, while gelatin is a protein in which no tryptophan is present. The explanation is self evident on this basis.

CONCLUSIONS

On a medium containing no organic nitrogen, no proteolytic enzymes are formed by bacteria.

On gelatin, casein, broth, agar, and peptone enzymes are formed which will digest both gelatin and casein.

The ferments obtained from mediums containing amino-acids as the only source of organic nitrogen show various actions. The ones from asparagin digest both gelatin and casein; on glycocoll, no caseases are liberated; and on tyrosin, no gelatinases.

Proteolytic enzymes are apparently formed to correspond to the different amino-acids present in the medium and will then attack these acids whether combined or free.

These enzymes are not preformed in the bacterial cell but are dependent on the content of the medium on which it grows.

The specificity of proteolytic enzymes is resident in the amino-acids composing the proteins and not in the proteins themselves.

Differences in the chemical constituents of mediums may account for many of the unverified "new strains" of bacteria, the differentiation of which is based on some minor phase of their action on culture mediums.

BLACKLEG TOXIN

T. P. HASLAM AND J. W. LUMB

From the Purity Biological Laboratories, Sioux City, Iowa

The production of toxin by *B. chauveaui* has long been a mooted question. Roux,¹ Duenschmann,² and later Leclainche and Vallee,³ reported the formation of toxic substances in cultures of blackleg. Leclainche and Vallee³ showed that filtrates of 5-day old cultures of blackleg grown on Martin's bouillon were able to kill guinea-pigs promptly in doses of 5 c.c., injected intraperitoneally. They did not succeed in immunizing guinea-pigs with this toxin, because doses that were not immediately fatal produced severe emaciation and death in 1 or 2 weeks. Leclainche and Vallee concluded from their experiments that the serum from horses immunized with cultures grown on Martin's bouillon, although strongly anti-infectious, is not antitoxic, because guinea-pigs made passively immune against infection by this serum regularly succumbed to the toxic effects of a dose of blackleg culture, with the same symptoms as those receiving only toxin. Later, Foth⁴ showed that cultures of blackleg produced soluble immunizing substances. Leclainche and Vallee, and also Foth, carefully established the identity of their cultures, especially distinguishing between *B. chauveaui* and "vibrion septique" (bacillus of malignant edema), and therefore their observations warrant careful consideration.

Recently Nitta,⁵ Eichhorn,⁶ and Kelser⁷ have made claims in regard to the production of toxin in cultures of blackleg, the latter holding that the immunizing properties of the filtrate are in direct relation to its toxicity, and that nontoxic filtrates have no value as immunizing agents. As no data are furnished by these authors in regard to the purity and identity of the strains used, their publications cannot be considered authoritative. The experiences of Schattenfroh and Grass-

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¹ Ann. de l'Institut. Pasteur, 1888, 2, p. 49.

² Ibid., 1894, 8, p. 40.

³ Ibid., 1900, 4, p. 202 and p. 590.

⁴ Ztschr. f. Infektionskrankh. der Haustiere, 1911, 10, p. 1; *ibid.*, 909, 201.

⁵ Jour. Am. Vet. Med. Assn., 53, n. s. 1918, 6, p. 466.

⁶ Ibid., 52, n. s., 1918, 5, p. 653.

⁷ Jour. Agri. Research, 1918, 14, p. 253.

berger with cultures of blackleg and other somewhat similar anaerobes emphasize the extreme necessity of careful purity and identity tests. Schattenfroh and Grassberger alone of the authors mentioned demonstrated that the poisonous substances occurring in their cultures were true toxins, capable of producing antitoxins, and not products of metabolism, and were justified in using the term toxin. However, von Hibler⁸ and others hold that in all probability Schattenfroh and Grassberger were confusing Fraenkel's bacillus with *B. chauveaui*.

PROPERTIES OF *B. CHAUVEAU*

Without attempting to go minutely into the differential diagnosis of blackleg, it would seem worth while to briefly recount some of the most important properties of *B. chauveaui*, especially because of some of the statements appearing in recent literature. Thus, one author⁹ finds that the filtrates prepared by a colleague were strongly alkaline. Eichhorn⁶ records that the meat added to his blackleg culture was digested. The filtrates made by certain laboratories have exceeding foul odors. According to von Hibler,¹⁰ blackleg produces an acid reaction, does not peptonize sterile meat in cultures, and does not produce a putrid odor. Th. Smith,¹¹ von Hibler,¹⁰ Leclainche and Vallee,⁸ and Foth,⁴ agree in every essential point as to the morphologic and cultural characteristics of *B. chauveaui*. Of the various reactions mentioned by these authors the following have proved especially useful in checking the purity and identity of cultures previous to their use in experimental work, and the strain of blackleg used in our experiments possessed these characteristics.

In peptone liver broth with cooked brain tissue a vigorous evolution of gas occurs within 24 hours, and the medium becomes moderately cloudy. In a few days evolution of gas ceases and the liquid part of the medium becomes as clear as before inoculation. There is no blackening of the medium and no formation of putrid odor even in cultures 2 weeks old or more. The cultures remain permanently acid. If the medium is highly alkaline at the start the organisms may not be able to continue growth until it is acid, but the alkalinity will be at least neutralized.

Emulsion of a 24-hour culture in dose of 0.1 cc will usually kill guinea-pigs which show at the point of inoculation a large area of hemorrhagic edema without putrid odor or evidence of peptonization or digestion of tissue. Other related pathogenic anaerobes produce lesions very similar and differentiation on this ground is not attempted. Smears made from the diaphragmatic surface of the liver after death with blackleg show slender rods singly or in pairs, rarely in fours. This furnishes a valuable means of differentiating from *B. edematis-maligni* which produces long chains on the serous membranes although its form in the muscle tissue may very closely resemble blackleg.

⁸ Article by Felix v. Werdt in *Handbuch der Pathogenen Microorganismen* by Kolle and Wassermann, 1912, 4, p. 902.

⁹ Berg, William N.: *Jour. Agri. Research*, 1918, 14, p. 264.

¹⁰ *Handbuch der Pathogenen Microorganismen* by Kolle and Wassermann, 1912, 4, p. 800.

¹¹ *Ztschr. f. Infektionskrankh. der Haustiere*, 1905, 1.

As a final test of the identity of a strain showing these characteristics, a test is made with a serum made by another laboratory. In our work 3 guinea-pigs each receive 0.6 cc of the blackleg serum made by Institut de Sérotherapie de Toulouse, Professors Leclainche and Vallee. Twenty-four hours later these guinea-pigs and 3 check guinea-pigs receive 0.5 cc each of an emulsion of the culture to be tested. If the guinea-pigs receiving the serum live and the checks die the identity of the blackleg strain is considered sufficiently established.

MEASUREMENT OF TOXICITY ON GUINEA-PIGS AND IMMUNIZATION OF CALVES

It was the plan of the present experiment to test the toxic powers on guinea-pigs of filtrates of blackleg cultures made in this and other laboratories, and to test their immunizing power on calves, in order to ascertain if any relation existed between toxicity on guinea-pigs and immunizing power for calves.

TABLE 1
MEASUREMENT OF TOXICITY ON GUINEA-PIGS OF BLACKLEG FILTRATES
FROM FOUR DIFFERENT LABORATORIES

Guinea-Pig	Injection	Blackleg Filtrate C C	Result
Group 1—Filtrate A			
1	Subcutem	10.0	Slight swelling
2	Subcutem	10.0	Marked swelling
3	Subcutem	10.0	Slight swelling
4	Subcutem	10.0	Slight swelling
Group 2—Filtrate B			
1	Intraperitoneal	5.0	Slight swelling
2	Intraperitoneal	5.0	Slight swelling
Group 3—Filtrate C			
1	Intraperitoneal	5.0	Marked swelling
2	Intraperitoneal	5.0	Marked swelling
Group 4—Filtrate D			
1	Subcutem	10.0	Severe swelling, sick
2	Subcutem	10.0	Marked swelling
3	Subcutem	10.0	Marked swelling
4	Subcutem	10.0	Marked swelling
5	Intraperitoneal	10.5	Marked swelling
6	Intraperitoneal	10.0	Slight swelling
7	Muscles of leg	5.0	Slight swelling
8	Muscles of leg	5.0	Slight swelling

TABLE 2

IMMUNIZATION OF CALVES AND TEST FOR IMMUNITY

The dose of filtrate was 5 cc and of virus 10 cc.

Group 1—Filtrate A		Group 3—Filtrate C	
1.....	Lived	1.....	Lived
2.....	Lived	2.....	Lived
3.....	Lived	3.....	Lived
Group 2—Filtrate B		4.....	Lived
1.....	Lived	Group 4—Filtrate D	
2.....	Died	1.....	Lived
3.....	Lived	2.....	Lived
4.....	Died	3.....	Lived

Ten nontreated calves receiving same dose of virus all died.

The filtrates tested were usually given subcutaneously in the region of the pectoral muscles and the adjacent axilla which allows 5 or 10 cc to be injected and absorbed with ease. As a check on the method of inoculation some animals were injected in the muscles of the leg, and some intraperitoneally. Table 1 gives a summary of the effect on guinea-pigs of blackleg filtrates from 4 different laboratories, and it is seen that all of the filtrates were nontoxic, none of them being able to produce death or serious symptoms in guinea-pigs when given in doses of 5 or 10 cc.

All of the calves in this experiment received 5 c c of blackleg filtrate each and after at least 2 weeks were tested for immunity by inoculation with a culture of blackleg carefully tested for purity as already detailed. The result of the test with calves is given in Table 2, from which it is seen that of 14 calves treated with filtrate only two were later susceptible to virus. Ten controls receiving virus alone, all died of blackleg. It will be noted that the filtrates from the 4 different laboratories possessed marked immunizing properties. It is not within the scope of this paper to compare the efficiency of the various filtrates, and no inquiry was made as to their method of production, so on that point no statement can be made. They were all believed by their makers to be efficient immunizing agents against blackleg, and this contention was in the main confirmed, in spite of the fact that they in no instance possessed any marked toxicity.

SUMMARY

Blackleg filtrates made by four different laboratories were non-toxic to guinea-pigs, and possessed marked immunizing properties in calves. The experiments indicate that the immunizing power of blackleg filtrates is not dependent on toxicity.

THE RAT-BITE FEVER SPIROCHETE, WITH COMPARATIVE STUDY OF HUMAN, WILD RAT AND FIELD VOLE STRAINS

SHIGERU KUSAMA, ROKUZO KOBAYASHI
AND KATSUYA KASAI

From the Kitasato Institute for Infectious Diseases, Tokyo

In 1915 Futaki, Takaki, Taniguchi and Osumi¹ discovered in a swollen lymph gland of a rat-bite fever patient a spirochete which they regarded as the cause of this disease. A month later, Ishiwara, Ohtawara and Tamura² succeeded in finding the same spirochete in a guinea-pig experimentally infected with rat-bite fever, transmitted by the bite of a wild rat. Subsequently Kitagawa, Kato, Abe and Mukoyama,³ Futaki and his associates,⁴ Kaneko and Okuda,⁵ and Row,⁶ in India, each group working separately, again demonstrated the existence of this spirochete, making use of specimens obtained from human beings; and Ido, Ito, Wani and Okuda⁷ showed that there is a specific relation between Futaki's spirochete and antibody in the blood of patients. *Spirochaeta morsus-muris*, as it was designated by Futaki and his associates,⁸ has since been proved beyond doubt to be the causative agent of the rat-bite disease occurring in Japan.

In addition to the investigations just noted, there have been published in Japan several papers on the rat-bite fever spirochete. A number of contradictory statements in these publications have led us to repeat certain experiments; moreover our interest in the morphologic similarity between this spirochete and that found in the blood of the field vole by Miyajima has made a comparative study of these strains advisable.

For this experiment we selected four strains of the spirochete, the human 1 and 2,* the wild rat and the field vole strains.

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* We are indebted to Dr. Ido for the human strain 1 and to Dr. Kitagawa for the human strain 2.

¹ Tokyo Igakukwai-Zasshi, 1915, 29; Jour. Exper. Med., 1916, 33, p. 249.

² Tokyo Igakukwai-Zasshi, 1916, 30; Jour. Exper. Med., 1917, 25, p. 45.

³ Igaku-Chuo-Zasshi, 1916, no. 126.

⁴ Futaki, K., Takaki, I., Taniguchi, T. and Osumi, S.: Tokyo Iji-Shinshi, 1916, no. 1976; Jour. Exper. Med., 1916, 33, p. 249.

⁵ Iji-Shimbun, 1916, nos. 951 and 952; Jour. Exper. Med., 1917, 26, p. 363.

⁶ Ind. Jour. Med. Research, 1917, 5, p. 386.

⁷ Jour. Exper. Med., 1917, 26, p. 377.

⁸ Futaki, K., Takaki, I., Taniguchi, T. and Osumi, S.: Jour. Exper. Med., 1917, 25, p. 33.

DISTRIBUTION IN THE ANIMAL BODY

Seventeen guinea-pigs, 6 wild rats and 13 white rats were each inoculated with one of the four strains and killed between 8 days and 4 months after inoculation. All the organs of the animals thus treated were then examined, the preparations being stained by Levaditi's old method.

Our investigation showed the distribution of the spirochete to be the same for each strain in each animal. In the early stages of the infection the spirochetes are found principally in the blood, but after 2 weeks a large number appear in the connective tissue which as time goes on is gradually increased. The following is the arrangement of the organs in order according to the number of organisms found; the subcutaneous and submucous connective tissue and muscle of the eyelids, lips, bridge of the nose and tongue, the intermuscular connective tissue of the heart wall, the capsules of the salivary and lymph glands, the adventitia of aorta and large arteries of the testicles, lungs, kidneys, etc., the spleen, the liver, the adrenal glands, the kidneys, the parenchyma of the salivary and lymph glands, etc.

As far as we know, there has been no previous record of the constant occurrence of large numbers of spirochetes in the adventitia of the aorta and large arteries within the visceral organs, nor of their occasional abundant occurrence in the endocardium of the heart and their concentration in the reticular connective tissue of the vascular sinus surrounding the follicle of the tactile hair of the upper eyelids and lips.

Notwithstanding many workers have stated that they have found this spirochete in the excretory ducts and the secretory tubules of the salivary glands, and in the tubules of the kidneys, we have been unable to find the organism in these tissues.

ROUTE OF EXCRETION

As the name indicates, rat-bite fever is caused by the bite of a rat, but it is important to ascertain whether or not the spirochete in question can be discharged in the saliva. Accordingly the saliva of the infected guinea-pigs, wild rats and white rats was examined directly by microscopic examination of preparations of saliva, and indirectly by inoculation of saliva solution obtained by washing the mouth cavity carefully with about 0.3 cc of salt solution. In no case could we detect the existence of spirochetes in saliva by the first method, but the inoculation of mice with the saliva of 7 infected animals, 2 guinea-pigs, 3 wild rats and 2 white rats, yielded positive results only in the case of the mice inoculated with the saliva of one of the wild rats. We do not consider this one positive result sufficient evidence that the disease is caused by the excretion of the spirochete in the saliva; or through the normal mucous membrane of the mouth cavity, as is held by Futaki and his associates.

The frequent excretion of this organism in the urine has also been reported by many investigators. But, in view of our failure to find the spirochete in the tubules of the kidneys, it seemed necessary to repeat the investigations with regard to the urine. Nineteen infected guinea-pigs were killed and the urine taken aseptically from their bladders, a syringe with a burning needle being used. We then inoculated from 1-10 mice for each guinea-pig using about 0.25 cc of urine per mouse. From those 19 cases, killed at various stages after inoculation, we obtained only two positive results. The excretion of this organism in the urine, therefore, seems not to occur as frequently as is generally believed and to be indeed rather rare.

The next hypothesis to be tested was that of the excretion of the spirochete in the bile. We examined the bile of 10 infected guinea-pigs by the same methods as those used in the case of the urine, but with no positive result in any case, as was also the case in the experiments of Futaki and his associates.

We were similarly unable to find microscopically the spirochete in the intestinal contents of a number of guinea-pigs, wild rats, and white rats. The intestinal contents of an infected guinea-pig and a wild rat were inoculated into 4 guinea-pigs, but without positive results.

MODE OF INFECTION

The frequency of the occurrence of this infection in wild rats is 21 to 186 (11.2%) according to Ishiwara and his associates, 5 to 84 (5.9%) according to Matsuzaki and his associates,⁹ 8 to 58 (13.8%) according to Tsuneoka¹⁰ and we detected it in 2 of 24 wild rats. These results show that this form of spirochetosis is prevalent among the wild rats in Japan. Moreover, there is now no doubt that the disease is transmitted by the bite of the spirochete-bearing wild rat. This was conclusively proved first by the experiments of Ishiwara and his associates and later by others. Our experiments showed 5 positive cases among 7 guinea-pigs bitten by an infected wild rat.

The question therefore arises as to how in such cases this spirochete is transmitted from its source in the body of the host if not through the saliva. The infected wild rat, and also the infected guinea-pig, usually becomes very irritable and furiously bites any objects coming in its way, very often suffering an abrasion and even bleeding in the lips or gums. The spirochete is then given an opportunity of escape from the submucous tissue, or the circulating blood, through the defective point in the mouth. It is possible, therefore, that the spirochete may be thus transferred by the bite of an infected rat to the body of a healthy rat or even a human being. Nevertheless, it was desirable to ascertain whether this spirochetosis can be produced experimentally by other modes of transmission, as so many workers have supposed.

1. By keeping the infected and normal animals together in the same cages, we did not obtain a single positive result among 11 guinea-pigs, which were divided into 6 groups, each group being put with from 2-5 infected guinea-pigs. The same experiment was tried with 29 normal mice, which were placed in 7 cages, each with from 5-15 infected mice. Only 2 positive cases were observed, each in a different cage. In these experiments, the observation lasted from 1 week to about 1 month after inoculation in the case of the mice and 2 or 3 months in the case of the guinea-pigs. From the foregoing results, it is natural to conclude that these 2 positive cases, found only among the mice, might have resulted from the bite of the infected mice, and it seems far from possible, in spite of the assertion to the contrary of Haga and his associates,¹¹ that the spirochete can be easily transmitted, especially in the case of guinea-pigs, through the normal skin, or by ectoparasites, such as lice, fleas and mites.

2. By feeding clot densely contaminated by spirochetes to 24 mice, we obtained 4 positive results. In most cases these mice were kept in separate cages, in order to prevent possible infection by biting; but the 4 positive

⁹ Matsuzaki, H., Yamamoto, M., and Imai, K.: Tokyo Iji-Shinshi, 1916, nos. 1980 and 1981.

¹⁰ Kyoto Igakukwai-Zasshi, 1917, 14, No. 4.

¹¹ Haga, I., Yoshizawa, K., Umemoto, E. and Hiroshige, J.: Nihon Eiseigakukwai-Zasshi, 1917, 13, no. 1.

results obtained occurred only among the mice which were put together and there were no positive cases among the mice which were separated. We found 2 positive cases among 14 similarly fed guinea-pigs.

3. By the instillation of blood containing large numbers of the spirochetes into the eye, we obtained only one positive case among 19 mice, which were kept in separate cages, and 4 among 14 instilled guinea-pigs.

From the results obtained in the preceding experiments in feeding and instillation of the organism, it seems hardly possible that it enters the animal body through the normal mucous membrane; for it is more than likely that the positive cases resulted from small abrasions. Therefore, contrary to the opinion of Haga and his associates, we are certain that, even in the case of guinea-pigs, this infection does not readily occur through the digestive tract.

We believe that the natural and most important source of infection among wild rats is the bite of an infected rat, having a wound in the vicinity of the mouth. Infections by other means, under natural conditions, occur very rarely, if at all.

SYMPTOMS IN THE INFECTED ANIMALS

As many workers have already proved, the spirochete easily infects such experimental animals as the mouse, white rat, wild rat, guinea-pig and monkey; but one perceives a constant clinical change only in the guinea-pig and monkey, the other animals continuing apparently healthy, in spite of the abundant increase of the organism in their blood.

With regard to the clinical changes in the guinea-pigs and monkeys, infected with the human, wild rat, or field vole strain, we observed that in the guinea-pig, whether the inoculated strain be the human, wild rat, or field vole, the most important and constant symptoms are decrease in weight and alopecia. About 1 month after inoculation, the alopecia begins to appear around the face and at last spread over the whole body. The fever also is a constant symptom in guinea-pigs inoculated with the human or wild rat strain, but it seldom occurs in the case of the field vole strain.

In all cases of monkeys, especially Japanese monkeys, which we used as the experimental animals, and which we inoculated with the human or wild rat strain, we observed fever after about 1 week of inoculation, but, after several successive paroxysms, it usually subsided. In these cases we always detect the spirochete in the blood, if not microscopically, by inoculation. On the contrary, there was no reaction either microscopically or clinically in any of the 3 monkeys inoculated with the field vole strain. In this case, we were unable to credit the monkey's susceptibility to the field vole strain until it was conclusively proved by the immunologic reaction.

IMMUNITY RELATIONS BETWEEN THE HUMAN, WILD RAT AND FIELD VOLE STRAINS

The fact that specific antibody appears in the blood of rat-bite fever patients and has a spirocheticidal or lytic action on the causative spirochete has been demonstrated by Ido and his associates and by others. We also have tried to produce immunity in the animals experimentally and have succeeded in immunizing the Japanese monkey alone, no result being obtained in the case of the guinea-pig, white rat and mouse. This finding is consistent with the fact that the number of spirochetes in the body of the guinea-pig, white rat and mouse does not decrease, even after several months of infection, but rather increases in the connective tissues. On the other hand, we were unable to find the spirochete in the bodies of Japanese monkeys killed after recovery.

Being unable to obtain the serum of a patient, we used for the present investigation the serums of infected Japanese monkeys: monkey 18 inoculated with the human strain 2, monkey 19 inoculated with the wild rat strain, monkeys 1, 15 and 10 inoculated with the field vole strain and monkey 47 inoculated with the wild rat strain killed by exposure for 30 minutes to a temperature of 56 C.

Examination by Dark-Field Illumination.—The serum to be tested is first drawn into a capillary pipet up to a given mark. The blood taken from the tail of a heavily infected mouse is measured in the same way and the equal quantities of blood and serum thus obtained are thoroughly mixed by repeatedly drawing them in and out of the pipet. The mixture is then transferred to a slide, and examined under the dark-field microscope. The actions of the immune serums on the human and wild rat strains are shown in the following table.

TABLE 1
ACTIONS OF THE IMMUNE SERUMS ON HUMAN AND WILD RAT STRAINS

Serum	5 Minutes	15 Minutes	30 Minutes	1 Hour	Result
Human strain 2 serum (monkey 18).....	Motility languid	Rest	Rest	Rest	Positive
Wild rat strain serum (monkey 19).....	Motility languid	Rest	Rest	Rest	Positive
Field vole strain serum (monkey 1).....	Motility active	Active	A little languid	Rest	Weak positive
Field vole strain serum (monkey 10)....	Motility active	Active	Active	Active	Negative
Field vole strain serum (monkey 15)....	Motility active	Active	Active	Active	Negative
Japanese monkey 47 serum.....	Motility active	Active	Active	Active	Negative
Normal Japanese monkey serum 1.....	Motility active	Active	Active	Active	Negative
Normal Japanese monkey serum 2.....	Motility active	Active	Active	Active	Negative

TABLE 2
ACTION ON THE FIELD VOLE STRAIN

Serum	5 Minutes	15 Minutes	30 Minutes	1 Hour	Result
Human strain 2 serum (monkey 18).....	Motility languid	Rest	Rest	Rest	Positive
Wild rat strain serum (monkey 19).....	Motility languid	Rest	Rest	Rest	Positive
Field vole strain serum (monkey 1).....	Motility active	Rest	Rest	Rest	Positive
Field vole strain serum (monkey 10)....	Motility active	Languid	Partly rest	Rest	Weak positive
Field vole strain serum (monkey 15)....	Motility active	Active	Active	Active	Negative
Japanese monkey 47 serum.....	Motility active	Active	Active	Active	Negative
Normal Japanese monkey serum 1.....	Motility active	Active	Active	Active	Negative
Normal Japanese monkey serum 2.....	Motility active	Active	Active	Active	Negative

The immune serums for the field vole strain have no action on the human and wild rat strains, as the control serums, except a slight action of the serum from monkey 1, while the immune serums for the human and wild rat strains have a distinct spirocheticidal action on their homologous strains.

The actions of these immune serums on the field vole strain are recorded in table 2.

Although the human strain and wild rat strain serums have a strong spirocheticidal action on the field vole strain, the field vole strain serums do not react in the same way to their homologous strain, except in the case of the serum from monkey 1.

TABLE 3
RESULTS OF EXPERIMENTS TO DETERMINE SPIROCHETICIDAL ACTION

Serum	Strain	Result
Human strain 2 serum (monkey 18)	Field vole strain	Positive
	Wild rat strain	Positive
	Human strain	Positive
Wild rat strain serum (monkey 19)	Field vole strain	Positive
	Wild rat strain	Positive
	Human strain	Positive
Field vole strain serum (monkey 1)	Field vole strain	Positive
	Wild rat strain	Negative
	Human strain	Negative
Normal Japanese monkey serum	Field vole strain	Negative
	Wild rat strain	Negative
	Human strain	Negative

TABLE 4
RESULTS OF EXPERIMENTS TO DETERMINE WHETHER OR NOT REINFECTION OCCURS

Monkey Number	Strain Injected at the First Inoculation	Strain Injected at the Second Inoculation	Interval Between First and Second Inoculation	Result
Monkey 18	Human strain 2	Human strain 2	68 days	Negative
Monkey 1	Field vole strain	Human strain 2	25 days	Negative
Monkey 10	Field vole strain	Human strain 1	80 days	Negative
		Human strain 2 (at the third)	132 days	Negative
		Human strain 2 (at the fourth)	150 days	Negative

Japanese monkey 47, inoculated with the killed rat strain, was infected typically by reinoculating the human strain.

To conclude, as far as examination of the slide is concerned, the field vole strain serum has no spirocheticidal action or only a slight one on the human and wild rat strains, and even on its homologous strain, while the human strain and wild rat strain serums always exert a strong spirocheticidal action on all these three strains.

Pfeiffer's Reaction.—Equal quantities of immune serum and infected blood are mixed and sodium citrate is added in a ratio of 1%. After being kept one hour at 37 C., the mixture is injected intraperitoneally into guinea-pigs. The result of this test is determined by the symptoms in the animal thus treated, by microscopic examination of its blood, and also, if necessary, by inoculation of the heart blood into mice.

The wild rat strain and human strain serums have a spirocheticidal action on all three strains, but the serum of the field vole strain has no positive action except on the field vole strain.

Examination by dark-field illumination and Pfeiffer's reaction, therefore, indicate the same fact, namely, that the field vole strain is apparently different from the other two strains, while the human and wild rat strains are evidently the same.

Reinfection Test in the Japanese Monkey.—We endeavored to ascertain whether or not reinfection occurs in the previously infected Japanese monkey on reinoculation of the human strain within a certain interval after the first inoculation.

The Japanese monkey, therefore, is not only made immune by the infection of the human strain, but it also develops immunity by the infection of the field vole strain, even enough to resist subsequent inoculation with the human strain.

We may conclude, therefore, from the foregoing immunological result, that the field vole strain is similar to both the human and the wild rat strains. The distinct difference in the virulence and immunizing power between the field vole strain and the others is probably due to the fact that, in passing through many generations of field voles, the spirochete decreases its toxophore and haptophore groups quantitatively.

Identity of *Spirochaeta morsus-muris* Futaki and *Spirillum minor* Carter, *Spirochaeta laverani* Breinl et Kinghorn, *Spirochaeta muris* Wenyon, etc.

Futaki and his associates regarded *Spirochaeta morsus-muris* as a new species, insisting that the presence of flagella and pathogenity in this spirochete differentiates it from the allied spirochetes referred to by other workers.

From table 5 as well as from the original figures of the workers, it is difficult to detect any difference in morphology, movement and pathogenity between Futaki's and the allied spirochetes, except for a slight difference of *Spirillum minor*.

As mentioned, Carter, Breinl and Kinghorn, and Wenyon could not prove the presence of flagella in their spirochetes, but it must be remembered that the process of dark-field illumination had not come into use in their time. This fact is especially noteworthy in the light of the subsequent discoveries of MacNeal, Mezincescu and Deetjen, who found flagella in their spirochetes by means of dark-field illumination.

Regarding pathogenity, there is also a slight distinction between Futaki's and the allied spirochetes. We must, however, again take into account the insufficiency of the animal experiments made by these workers and the variation in the virulence of spirochetes, which might be due very often to the difference in the species of their hosts, as has just been indicated in the case of the field vole strain.

It would seem therefore that *Spirochaeta morsus-muris* Futaki is, in all probability, similar to *Spirillum minor* Carter, *Spirochaeta laverani* Breinl et Kinghorn, *Spirochaeta muris* Wenyon, etc.

SUMMARY

In the infected wild rat, white rat and guinea-pig, the spirochete of rat-bite fever, in the early stages of the infection, are detected principally in the blood, but after 2 weeks a large number appears in the connective tissues, and as time goes on this number is gradually

TABLE 5

MORPHOLOGY AND PATHOGENESIS OF RAT-BITE FEVER SPIROCHETES

Name of Spirochete	Habitat	Length, μ	No. of Turns	Flagellum	Movement	Pathogenity
<i>Spirillum minor</i> ¹² Carter (1887)	Indian rat (<i>Mus decumanus</i>)	5-9	4-8	No flagellum (in aniline-stained preparation)	"Very active and tolerably sustained, consisting of rotation round the long axis, propulsion either forward or backward, and occasionally an energetic lateral twisting or lashing."	Not transmissible to the rat and monkey (macacus)
<i>Spirochaeta laverani</i> ¹³ Breinl et Kinghorn (1906)	White mouse and wild mouse (<i>Mus musculus</i>)	1.8-3.75	2-4	No description of flagellum	"Actively motile, and shows both a movement of the spiral turns and a progressive movement of the whole organism."	Transmissible to the mouse and rat ¹⁴
<i>Spirochaeta muris</i> ¹⁵ Wenyon (1906)	Brown mouse	3-7	2-6	"All its movements seem to indicate the presence of flagella though these were never actually seen."	"Moving along very rapidly, and turning on its long axis, it will suddenly come to rest and as suddenly dart off in an opposite direction."	Transmissible to the mouse and young rat, but not to the guinea-pig nor the adult rat
<i>Sp. muris</i> , var. <i>Virginiana</i> ¹⁶ MacNeal (1907) <i>Sp. muris</i> , var. <i>Galatziana</i> ¹⁷ Mezincescu (1909)	Wild rat (<i>Mus decumanus</i>)	1.75-3.55	1 $\frac{1}{4}$ -3 $\frac{1}{2}$	One at each end	"It appears as an elastic spiral rapidly darting about, as often with one end forward as the other, forth and back, rotating on its long axis as it goes. . . . At times a lashing movement of the whole body is seen."	Transmissible to the wild rat, white rat and house mouse (<i>Mus musculus</i>)
Spirochete found in the ¹⁸ mouse cancer by Deetjen (1908)	Mouse	1.5-5	1-5	One at each end	"Actively motile, and shoots to and fro, turns on both the long and transverse axes and turns over."	Transmissible to the mouse
<i>Spirochaeta morsusmuris</i> Futaki, Takaki, Taniguchi et Osumi (1915)	Human being	1.5-6	1.5-6	One at each end	"The movements are spasmodic, usually spiral, and freely pass backwards and forwards."	Transmissible to the human being, monkey, guinea-pig, wild rat, white rat and mouse, especially pathogenic for the first ³

increased. That is, this spirochete is always distributed numerously in the subcutaneous and submucous tissues of the eyelids, lips, bridge of the nose, and tongue, and is especially abundant in the reticular connective tissue of the vascular sinus surrounding the follicle of the tactile hair of the upper eyelids and lips. It is also usually, if not always, found abundantly in the capsules of the salivary and lymph glands, in the heart wall, in the adventitia of the aorta and large arteries within the visceral organs, and sometimes in the endocardium of the heart. It can also be detected in the spleen, the liver, the adrenal glands, the kidneys, the parenchyma of the salivary and lymph glands, etc.

The spirochete is neither excreted through the saliva from the salivary gland nor mixed into the saliva through the normal mucous membrane of the mouth cavity from its submucous source.

The excretion of the organism in the urine is comparatively rare.

The spirochete has never been detected in the intestinal contents of wild rats and guinea-pigs or in the bile of guinea-pigs.

It is transmitted to a healthy animal through a wound caused by the bite of an infected animal and we have reason to believe that in order to be transmitted it passes from the submucous source or from the circulating blood, through an abrasion in the mouth, such as frequently occurs in the wild rat at the time of the bite.

By keeping the infected and healthy animals in the same cage, we found no positive case among 11 guinea-pigs and only two positive cases among 29 mice. These two positive cases might have resulted from the bite of the infected mice. So it is doubtful that this spirochetosis can spread among the animals merely by their living together, and it is also doubtful that transmission can take place by means of such ectoparasites, as lice, fleas, mites, etc.

By feeding experiments, we could find only two positive cases among 14 guinea-pigs and four among 29 mice, so that it is natural to suppose that the infection in the positive cases resulted from an injured surface in the alimentary canal rather than by transmission through the normal mucous membrane.

By instillation of the infected blood into the eye, we proved only one positive case among 19 mice, and four among 14 guinea-pigs. Thus it seems hardly possible that this organism enters the animal body through the normal conjunctiva, at least, in the case of the mouse.

The Japanese monkey, guinea-pig, wild rat, white rat and mouse are susceptible to all three strains of this spirochete. The infected guinea-pig shows the rise of temperature, decrease in weight, alopecia, etc., while the inoculated wild rat, white rat, and mouse are apparently healthy, in spite of the abundant increase of the organism in their blood. The monkey inoculated with the human or wild rat strain also shows the pyrexia of relapsing type, but if the field vole strain is used, there is no fever.

The immune serum of the Japanese monkey treated with the human or wild rat strain exerts a spirocheticidal action on all three strains, while the field vole serum has only a slight action on the field vole strain, but none on the other two.

The Japanese monkey, recovered from the infection, even if it be caused by the field vole strain, does not show any further symptoms after being reinoculated with the human strain.

The spirochete under discussion does not undergo any considerable variation in virulence and immunizing power, in passing through the body of the human being, monkey, guinea-pig, wild rat, white rat, mouse, etc., but in passing through the body of the field vole, it seems to decrease its toxophore and haptophore groups quantitatively.

Our experiments prove that the human, wild rat and field vole strains all represent the same species.

We believe that *Spirochaeta morsus-muris* Futaki is, in all probability, similar to *Spirillum minor* Carter, *Spirochaeta laverani* Breinl, *Spirochaeta muris* Wenyon, etc.

¹² Sci. Mem. Med. Off. Arm. India, 1887, 3, p. 45.

¹³ Lancet, London, 1906, 2, p. 651.

¹⁴ Mem. XXI of Liverpool School Trop. Med., 1906, 53.

¹⁵ Jour. Hyg., 1906, 6, p. 580.

¹⁶ Proc. Soc. Exper. Biol. and Med., 1907, 4, p. 125.

¹⁷ Compt. rend. Soc. de biol., 1909, 66, p. 58.

¹⁸ München. med. Wchnschr., 1908, 55, p. 1167.

EXCLUSION OF AIR IN THE CULTIVATION OF THE GONOCOCCUS

E. H. RUEDIGER

From the Pathological Laboratory of the Bismarck Hospital, Bismarck, N. D.

During a general investigation on the cultural requirements of the gonococcus in which all aerobic methods proved more or less uncertain and unsatisfactory, exclusion of air was resorted to. A gram-negative diplococcus which did not grow on ordinary culture mediums, which did not grow on the special medium unless the air was excluded, and which was obtained from clinical gonorrhea was called gonococcus.

For the work 500 gm. of minced veal were boiled in a liter of distilled water for an hour. After cooling it was strained through cloth; with normal solution of sodium hydrate the filtrate was made neutral to phenolphthalein, and 15 gm. of bacto-agar added. After boiling until the agar was dissolved it was divided into 5 different portions — A, B, C, D and E. Portion A was tubed as it was. Portions B, C, D and E received bacto-peptone enough to make 1%. In addition to peptone, portion C received sodium chlorid enough to make 0.5%; portion D received glycerol enough to make 3% and portion E received dextrose enough to make 1%. The mediums were autoclaved in test tubes. After cooling sufficiently each portion was subdivided into portions 1 and 2. Portion 1 received human blood which had been heated to about 56 C. for 30 minutes, and portion 2 received human blood which had not been heated. About 1 cc of blood was added to 10 cc of medium. The blood was well mixed with the medium, the tubes were placed in a slanting position and the medium allowed to solidify. After the medium had solidified each tube was stoppered air-tight with a sterilized cork stopper.

On comparing these different mediums it was found that the gonococcus did not grow well on those containing unheated blood.* It grew in all tubes which contained heated blood provided the stopper was inserted immediately after inoculation. Without the stopper growth did not take place as a rule. The largest growths — growths almost as luxuriant as those of typhoid bacilli on ordinary nutrient agar — were obtained on portions B 1, C 1 and D 1, portion C 1 usually containing the best growth. Portion A 1 usually had a fair growth, while portion E 1 yielded poor growth.

Up to the present time the method has been used on three cases.

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* Since this report was written very good subcultures have been obtained on medium containing unheated human blood.

1. A young woman was operated on by Dr. N. O. Ramstad for a probable appendicitis. At operation acute right-sided salpingitis and local peritonitis was found. The right tube was removed. Some of the seropurulent contents were streaked on the various mediums. Growth occurred on all tubes except those which contained the unheated human blood. The best growths developed on tubes containing peptone, peptone and salt, and peptone and glycerol, the first two being superior to the last. Microscopic examination showed the growth to be a pure culture of a gram-negative diplococcus. The diplococcus did not grow on ordinary mediums, it did not grow on the special mediums without exclusion of air, it was obtained from clinically gonorrheal material, and hence was called gonococcus.

2. A woman giving a history of acute gonorrheal infection 3 months previous to examination was admitted to the hospital because of a painful, swollen knee. About 10 cc of slightly turbid fluid were withdrawn from the knee joint by Dr. N. O. Ramstad. Some of the fluid was streaked out on the surface of the various mediums; growth occurred in all tubes which contained heated human serum. No growth appeared in the tubes containing unheated human serum. The growth in the tubes with heated human serum was composed of a gram-negative diplococcus somewhat irregular in size and shape; it did not grow on ordinary mediums; it did not grow on the special mediums without exclusion of the air; it was obtained from clinically gonorrheal material, and hence was called gonococcus.

3. A young man complaining of acute urethritis consulted Dr. Schoregge. The pus contained many characteristic, gram-negative intracellular diplococci. Some of the pus was streaked out on the surface of the various mediums. Growth appeared on all the mediums containing heated human serum; the best growths being on the mediums containing peptone, peptone and salt, and peptone and glycerol. The mediums with unheated human serum failed to show any growth. The growth which appeared on the medium with heated human blood was composed chiefly of diplococci, very few contaminating colonies being present. The diplococcus was irregular in shape and size, gram-negative, it did not grow on ordinary mediums nor on the special mediums without exclusion of the air; it was obtained from clinically gonorrheal material, and hence was called gonococcus.

SUMMARY

Cultures of gram-negative diplococci have been obtained from gonorrheal material on medium composed of veal broth, made neutral to phenolphthalein, agar, salt, peptone and 10% human blood which had been heated to 56 C. for 30 minutes. Little or no growth was obtained on medium containing unheated human blood. On medium without salt the diplococci grew about as well as on the medium with salt. The addition of glycerol or dextrose seemed to be unfavorable as was also the omission of peptone. The culture tubes had to be stoppered air-tight. The growth obtained was nearly as luxuriant as that of *B. typhosus* on ordinary nutrient agar and so far has been uniformly successful.

THE GERMICIDAL POWER OF ANTISEPTIC OILS AND OF SUBSTANCES DISSOLVED IN OIL*

PHILIP D. McMASTER

PHILADELPHIA

It has been known definitely since the time of Koch that certain essential oils have considerable disinfectant value. It has been assumed since his time also that substances which have disinfectant value when in watery solution are largely rendered inactive by solution in oil. This assumption arose as a wide generalization from what was actually observed by Koch in only one case, namely, that of phenol in vegetable oils, such as olive oil or cotton seed oil. In this case the validity of the observation is undoubted, but there have been a very few practitioners who have believed that in spite of the test tube indications the best way to apply phenol as a wound disinfectant is in solution in vegetable oil. The recent application by Dakin⁷ and his associates, of solutions of a water-insoluble oil-soluble substance (dichloramin-T) to the treatment of wounds indicates that from the practical point of view the question of the usefulness of disinfectants in oil is by no means closed. As a consequence of these general considerations, I undertook to study these questions anew.

The recognized methods of determining quantitatively the antiseptic value of substances presuppose water solubility. The immiscibility of water and oil prevents the use of broth culture (or the suspension of bacteria in salt solution) in the testing of an oily substance, or of material which has been in contact with an oil. It is doubtless for this reason — the physical difficulty of testing by standard methods — that the many new antiseptic oils and oil-soluble antiseptics are accepted solely on the basis of clinical experience and not on that of known germicidal power. The value of such oils has not up to the present been expressed in terms of a known standard, comparable to the "phenol coefficient" of water-soluble antiseptics.

Face to face with the necessity of deciding accurately the antiseptic power of a large number of oils the need for a preliminary investiga-

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* An investigation of disinfectants including certain oily solutions under the direction of Drs. Paul A. Lewis and A. N. Richards, made at the request of the medical committee of the Council of National Defense and carried out in the John Herr Musser Department of Research Medicine, University of Pennsylvania.

tion bearing on the possibility of determining the disinfectant power of any oil or substance dissolved in oil with sufficient accuracy to permit of quantitative expression became apparent.

The literature of this subject is scanty and shows that the favorite test, under these circumstances, has been that of the silk thread with dried anthrax spores. Koch¹ studied by this method the bactericidal properties of the essential oils, using them in full strength and expressing their power in terms of time. The killing times for bacteria of oils, such as oil of turpentine and eucalyptol, have been tested by Laubenheimer² in like manner.

Other observations are those of Kronig and Paul,³ Cadéac and Meunier,⁴ and Chamberland.⁵

My preliminary experiments were made with dried anthrax spores on silk threads, from which the oil was removed, after the test period, by washing with various substances capable of dissolving the oil, as alcohol, ether, chloroform, xylol, etc., but the results were so irregular and conflicting that this method was discarded as unsatisfactory.

I then approached the matter by planting *B. typhosus* on the surface of rather dry slants of nutrient agar, covering the growth with the oil or oily solution to be tested. After a suitable period of exposure the oil was poured off, the tubes were filled with sterile salt solution to float up the remaining oil droplets; this was again poured off and the viability of the remaining growth was tested by transplantation to fresh medium. Broth was chosen as a medium for this test transplantation with the idea that any oil remaining with the bacterial mass would be more certainly separated than would be true if agar slants were again used.

In the standard methods for determining the efficiency of disinfectants, known generally as either the Rideal-Walker or the Hygienic Laboratory test, the final expression is based on the concentration required to kill a definite number of bacteria in a certain time. The time for convenience is made very short, 5 minutes and 15 minutes being the accepted periods.

It was obvious that the test in hand could not be worked with these periods. The uneven thickness of the growth involving irregularities of penetration and the loss of time in washing off the oil must

¹ Mittheil. a. d. K. Gesundheitsamte, 1881, 1, p. 234.

² Allgemeine Bakteriologie und Sterilisationslehre für Aerzte und Pharmazeuten, 1915.

³ Ztschr. f. Hyg., 1897, 25, p. 5.

⁴ Ann. de l'Inst. Pasteur, 1889, 3, p. 317.

⁵ Ann. de l'Inst. Pasteur, 1887, 1, p. 153.

necessarily be compensated for by extending the time of exposure. Twenty-four hours seemed most convenient and was found in practice to give consistent results.

As a preliminary, experiments were made to determine the influence of the age, extent, and thickness of the culture upon the concentration required to kill. The time of exposure was 24 hours, and the temperature 37.5 C. Cultures were prepared of certain well defined types, as follows:

In type 1 the growth was a narrow smear with the base extending low enough to touch any water of condensation that might be in the tube. In type 2 the growths were mere spots. In type 3 the growth was smeared widely at the bottom in order to come in contact with any water of condensation which, collected at the base of the tube, might interfere with results. In type 4 the growth was kept well above any such water. Type 5 was similar to type 4 except that the surface of the agar was purposely cut deeply. In types 1 to 4 the surface of the mediums was not intentionally broken.

About 20 tubes of each type were planted, and after 24-48 hours' growth the tubes were filled with paraffin oil containing various percentages of phenol.

Of the 48-hour cultures none were killed by 0.2%, but all were killed by 0.6% phenol. The general results indicate that the killing concentration varies within a narrow range, between 0.2 and 0.6%. Likewise in the 24-hour cultures, in no case did 0.2% kill, but in about half of the tubes 0.4% killed, and in all cases 0.6% killed.

The most resistant cultures were those in which the surface had been intentionally wounded, the growth being therefore rather deep, and presumably protected from the action of the disinfectant.

When growth was obtained with 0.4% it occurred only in types in which the agar surface was wounded. In all other experiments in which the surface was not wounded death occurred at 0.4%.

These experiments therefore show that differences in the characteristics of the growths do not influence the action of the disinfectants if the surface is not injured. Control tubes in which paraffin oil was allowed to cover the growth for one day and two days gave growth after transplantation showing that the oil alone does not kill.

With this evidence at hand the next step was to determine the most suitable type of growth.

Over 100 tubes were planted of two types, and cultures of 24 hours were used. The two types were a simple streak, well above the bottom of the tube (type 4), and two dots also well above the water of condensation (type 2).

The three substances tested in this way were phenol, paracresol, and guaiacol. The results are shown in Table 1.

The minimal killing concentration of phenol is 0.4%; of paracresol, 0.2-0.3%, and of guaiacol 0.8-1%.

TABLE 1
TESTS MADE WITH THREE SUBSTANCES WITH TWO TYPES OF GROWTH

Substance Tested	Type of Growth	Dilution of Disinfectant in Paraffin Oil									
		0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	1.0
Guaiacol	IV*		++ ++ ++		++ ++ ++		++ ++ ++		— — +		— — —
	II		++ ++ ++		++ ++ ++		++ ++ ++		— ++ ++		— — —
Paracresol	IV	++ ++ ++	— — —	— — —	— — —		— — —				
	II	++ ++ ++	++ — ++	— — +	— — —		— — —				
Phenol	IV		++ — ++	— — —	— — —	— — —	— — —				
	II		++ — ++	— — —	— — —	— — —	— — —				

* In each test three tubes were used. + = growth; — = no growth.

It will be noted that the irregular results with paracresol and guaiacol occurred only in type 4 cultures, in which it is possible the surface of the mediums was punctured at time of inoculation. In all type 2 cultures the results were uniform.

With this experience a technic was adopted as follows: Agar slants, well drained of water of condensation, were inoculated with *B. typhosus* over an area not more than one centimeter and well above the middle of the tube. They were allowed to incubate 24 hours. The oily solutions were then poured over these so as to entirely cover the agar and the tubes allowed to stand 24 hours in the incubator at 37.5 C. The oily solutions were then poured off and the tubes twice washed out carefully with sterile salt solution. For this purpose an inverted wash bottle with a long pipe and a spring clamp on the tubing above a

glass beak served the purpose well. The growth was thus thoroughly washed on the surface of the agar and a transplant made to a tube containing 8 c.c. standard broth. Readings were made of these tubes after 24 and 48 hours' incubation.

For all purposes the 24 hour reading is satisfactory. In the total number of experiments only 4 tubes found negative at 24 hours became positive at 48 hours.

In order to determine whether phenol, dissolved in paraffin oil, would serve well as an arbitrary standard, in the testing of oily disinfectants, as it has in the testing of watery solutions, the following tests were made.

Solutions of phenol, paracresol, and guaiacol were made in paraffin oil and in water and tested simultaneously. The testing of the three substances in oil was according to methods previously outlined. In like manner the watery solutions were poured over agar tubes and tested as were the oil substances. Lastly, determinations of the phenol coefficients of the three substances were made by the Anderson-McClintock⁶ method. The results follow:

			Coefficients
In paraffin oil	phenol	kills at 0.4%; not at 0.3%	= 1.00
	guaiacol	kills at 0.8%; not at 0.7%	= 0.475
	paracresol	kills at 0.3%; not at 0.2%	= 1.55
In water	phenol	kills at 1.0%; not at 0.9%	= 1.00
	guaiacol	kills at 1.4%; not at 1.3%	= 0.69
	paracresol	kills at 0.6%; not at 0.5%	= 1.73
By the Anderson-McClintock method		phenol	coefficient = 1.00
		guaiacol	coefficient = 0.75
		paracresol	coefficient = 1.55

Or, more clearly expressed:

The coefficients of	By Anderson-McClintock method	In paraffin	In
		oil	water
Phenol	1.00	1.00	1.00
Guaiacol	0.75	0.475	0.69
Paracresol	1.55	1.55	1.73

These results seem to justify the use of the typhoid agar method in the testing of oils.

With the method thus elaborated, a series of experiments were made, many of which were repeated, to determine the value of certain disinfectants in oil and of certain proprietary oily disinfectants. Among various oils tested as a menstruum were Diamond Oil, Eclipse White Spindle Oil, and Rose White Oil brands of mineral oil

⁶ The Determination of the Phenol Coefficient of Some Commercial Disinfectants, Hygienic Laboratory Bulletin 82, 1912.

furnished by the Atlantic Refining Company. Experiments showed that the action of phenol in all these oils is the same. The difference in the composition of the oil has no apparent effect; 0.4% phenol kills in all cases, 0.3% is the border line, and 0.2% fails to kill in all cases. Control tubes in which the oils alone were used, gave growth in all instances.

Substance Tested	Lethal Point, %	May Kill, %	Does Not Kill, %	Coefficient
Phenol in paraffin oil.....	0.4	0.3	0.2	1.00
Iodin in paraffin oil.....	0.4	0.3	0.2	1.00
Phenol in cotton seed oil.....	2.5	2.0	1.0	0.16
Paracresol in paraffin oil.....	0.25	0.2	0.1	1.55
Guaiacol in paraffin oil.....	0.9	...	0.8	0.44
Proprietary preparation No. 1 in paraffin oil	10.0	...	5.0	0.04
Proprietary preparation No. 2 in paraffin oil	6.0	5.0	4.0	0.06
Proprietary preparation No. 3 in paraffin oil	10.0	7.5	5.0	0.04
Proprietary preparation No. 4 in paraffin oil	2.0	...	0.5	0.2
Dichloramin-T in 10% chlorinated eucalyptol in chlorinated paraffin oil.....	0.05	...	0.03	8.00
Dichloramin-T in plain paraffin oil.....	0.4	...	0.2	1.00

In all these experiments controls were used in which plain paraffin oil was poured over the agar slants. In all such, growth was obtained after 24 hours.

Experiments with menthol, camphor and turpentine resin showed that these did not kill in the highest concentrations that could be obtained.

As a preliminary to a series of tests of dichloramin-T a few experiments were made on the bactericidal properties of paraffin oil, eucalyptol, chlorinated paraffin oil and chlorinated eucalyptol, the results of which follow:

Paraffin oil alone, growth after 24 and 48 hours exposure.

Eucalyptol plain, growth after 24 and 48 hours exposure.

Ten per cent. eucalyptol in chlorinated paraffin oil, growth after 24 hours, kills after 48 hours' exposure.

Eucalyptol partially chlorinated, kills in 24 hours (growth in 8 hours).

Eucalyptol completely chlorinated, kills in 8 hours.

Dichloramin-T was then dissolved in chlorinated paraffin oil containing 10% chlorinated eucalyptol. Dilutions were always made with a 10% chlorinated eucalyptol in chlorinated paraffin oil, in order to keep the percentage of eucalyptol constant. Two sets of observations were made, and it was found that dichloramin-T in 0.05% kills, but not always 0.03%.

In this experiment, tubes covered with 10% eucalyptol dissolved in chlorinated paraffin oil but without dichloramin-T showed growth. This growth, however, was more scanty than that obtained with chlorinated paraffin oil alone.

In other tests in which dichloramin-T was added to plain paraffin oil, no growth was obtained at 0.4 and good growth at 0.3, giving the nonchlorinated combination a coefficient of 1, but reinforced with chlorinated paraffin oil a coefficient of 8.

In a final set of tests an attempt was made to reduce time of exposure. It is, of course, probable that a chlorin-containing substance as dichloramin-T in high dilution might exert its action in a shorter time than 24 hours and hence in determining the minimal killing concentration of such a substance a 24 hours' test would be inadequate. A comparison of the time reaction of dichloramin-T and phenol is presented in table 2 and shows that this is the case. In 6 hours the dichloramin has exerted its full action while the phenol required over 12 hours. The coefficient of dichloramin-T would therefore be so much the higher.

TABLE 2
TIME EXPERIMENT WITH PHENOL AND DICHLORAMIN-T IN PARAFFIN OIL

	Phenol					Dichloramin-T	
	0.6	0.5	0.4	0.3	0.2	0.06	0.05
3 hours		++ ++	++ ++	++ ++	++ ++	++ ++	++ ++
6 hours	— —	— —	++ ++	++ ++	++ ++	— —	— —
12 hours	— —	— —	— —	++ ++	++ ++	— —	— —
24 hours	— —	— —	— —	— ++	++ ++	— —	— —

Despite these results we believe it preferable in routine work to base results on the minimal killing concentration at 24 hours' exposure.

CONCLUSIONS

A method which may provisionally be regarded as satisfactory for determining quantitatively the germicidal power of antiseptic oils and substances dissolved in oil, has been developed.

Phenol dissolved in mineral oil has germicidal value approaching that of its value in water (under the limited conditions of our test)

and can serve as arbitrary standard for comparison when testing the activity of other oils and oil-soluble substances.

The method as so far developed is defective in that any advantage which a substance might possess in the shape of rapidity of action is largely masked by the 24-hour period of exposure used. Our work indicates that consideration given to this factor may lead to a limited degree of improvement in the method as here described.

HEMOLYTIC STREPTOCOCCI IN THE FAUCIAL TONSIL AND THEIR SIGNIFICANCE AS SECONDARY INVADERS

I. PILOT AND D. J. DAVIS

From the Department of Pathology and Bacteriology, University of Illinois, College of Medicine, and from the Cook County Hospital, Chicago

The recognition of hemolytic streptococci in recent measles epidemics as the chief cause of the fatal complications of empyema and bronchopneumonia has led many observers to investigate the frequency and habitat of these organisms both in patients and in healthy individuals. As a result, many data, some of a confusing nature, have been rapidly accumulating. In the winter of 1917 numerous reports on the frequency of the hemolytic streptococcus in the oropharynx were published from the army camps where streptococcal bronchopneumonia and empyema were prevalent. Levy and Alexander¹ found hemolytic streptococci in swabs of the pharynx in 14.8% of 489 new recruits, while in a company of healthy men at camp for six months they were found in 83.2%. Irons and Marine² found hemolytic streptococci in the pharynx of 70% of healthy men in the latter part of the epidemics of acute respiratory infections at Camp Custer. Fox and Hamburger³ reported the same organism in the pharynx of 15% of a group of normal men early in the measles epidemic and six months later in 83% of a similar company. Cole and MacCallum⁴ found the streptococci in the pharynx of 21.4% of individuals isolated with suspected tuberculosis. It is evident from these data that it is important to know the incidence and variations of hemolytic streptococci in normal and diseased throats.

The voluminous work prior to 1903 on the frequency of streptococci in the normal mouth is of little value because a satisfactory medium for differentiating the viridans types of streptococci from the hemolytic was not employed until the introduction of the blood-agar plate method.

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¹ Jour. Am. Med. Assn., 1918, 70, p. 1827.

² Ibid., p. 687.

³ Ibid., p. 1758.

⁴ Ibid., p. 1147.

Brailorsky-Lounkevitz,⁵ in a study of the flora of the normal mouth, noted the appearance of the *Streptococcus pyogenes* in one infant within 10 hours after birth and in another within 16 hours. Ruediger⁶ found hemolytic streptococci in 59% of 51 normal throats. Smillie⁷ described streptococci of the hemolytic type in 50% of 100 normal throats. In a series studied by us to be referred to later, hemolytic streptococci were found in 58% of 24 normal individuals. In all of these studies swab cultures were made from the pharynx or the surface of the tonsils.

Some years ago one of us⁸ noted that hemolytic streptococci occurred frequently in pure or predominating numbers in the crypts of 55 of 61 pairs of excised tonsils showing simple hypertrophy, and similarly in 31 of 38 tonsils from cases of arthritis and endocarditis. It was at that time indicated that swab cultures were of limited value in finding hemolytic streptococci. The statement was also made "that hemolytic streptococci may occur in the crypts and on the surface of tonsils not only in ordinary tonsillar infections, but also in normal throats; that these streptococci often cannot be distinguished from the streptococci found in the tonsils of patients suffering from joint troubles."

In a more recent study⁹ of streptococci in tonsillar crypts it was noted that hemolytic streptococci were present in predominating numbers in cultures from the epithelial surfaces lining the crypts of excised hypertrophied tonsils, whereas in the actinomyces-like granules often found in the crypts streptococci of the viridans type predominated. Just recently Nichols and Bryan¹⁰ report that they found hemolytic streptococci in the crypts of 75% of 100 pairs of extirpated tonsils, often in pure and predominating numbers, and in 28% of swab cultures of normal throats as compared with 50% of direct crypt cultures.

In the present work a study was made of swab cultures of the pharynx and tonsillar surfaces just before extirpation, and from the crypts of the excised tonsils from the same individuals immediately after extirpation with particular reference to the frequency and predominance of the hemolytic streptococcus. The patients, 100 in all,

⁵ Ann. de l'Institut. Pasteur, 1915, 29, p. 379.

⁶ Jour. Infect. Dis., 1906, 3, p. 755.

⁷ Ibid., 1917, 20, p. 45.

⁸ Davis, D. J.: Jour. Infect. Dis., 1912, 10, p. 148.

⁹ Pilot, I., and Davis, D. J.: Jour. Infect. Dis., 1918, 23, p. 562.

¹⁰ Jour. Am. Med. Assn., 1918, 71, p. 1812.

were chiefly children from 5 to 16 years of age who presented hyperplastic but not markedly diseased tonsils. Examination revealed no evidences of recent acute inflammation, fever or subjective symptoms of sore throat. The tonsils probably represent a closer approach to the normal than any other type of tonsil for which enucleation is indicated. Five adults who gave a history of repeated attacks of sore throat are included in the series. Grossly, the tonsils, when examined after excision, showed little or no change excepting that of hyperplasia. Frequently, firm actinomyces-like granules were found in the crypts. Pus was seldom encountered, but not infrequently the crypts contained a small amount of a thin, slightly turbid exudate. In a few instances soft yellow masses filled a crypt which in smears revealed amorphous material, often cholesterol crystals, and few or no bacteria.

Three separate swabs were applied, one to the pharynx, and one over each tonsillar surface, immediately before administration of the anesthetic. Cultures were made from these swabs within 1-4 hours. The tonsils, each wrapped separately in sterile gauze, were obtained immediately after extirpation. Macerated tonsils were discarded because of the impossibility of obtaining uncontaminated cultures of the crypts. Plain extract agar with a reaction of 0.3 was melted and defibrinated human blood added in proportion of 1 of the latter to 10 of the former. The melted blood agar tubes were inoculated directly with the swabs and poured into petri dishes. Each tonsil was incised cleanly with a sterile knife and the bottom of the crypts exposed. Material was collected from 3 or 4 crypts on a platinum loop and introduced directly into the melted blood-agar tubes which were poured into plates. Usually a dilution was made of the cultures both from the swabs and the tonsils. The plates were incubated aerobically at 37.5 C., and were observed at the end of 24 and 48 hours. Readings were made from the first plate in most instances. The dilutions were reserved for the isolation of pure cultures. In the study of the plates particular attention was given to the hemolytic colonies characteristic of streptococci as differentiated from the nonhemolytic streptococci which grew as small grayish-white colonies surrounded by a green or grayish-green zone. Staphylococci, diphtheroids and other organisms which occasionally cause hemolysis were eliminated by direct smear and study of subcultures.

The hemolytic streptococci grew as small round, discrete, grayish-white colonies surrounded by a clearly defined zone of hemolysis 2 to 4 mm. wide, which microscopically showed no red blood corpuscles. The colonies correspond to the Beta type of Smith and Brown.¹¹ From one patient similar colonies with clearly defined zones of hemolysis only 1-1.5 mm. wide were encountered. Occasionally colonies appeared in 24 hours with a green zone, and in 48 hours formed an indistinct narrow zone of hemolysis outside of the green zone. These colonies were classified under the nonhemolytic streptococci. Subcultures of the hemolytic streptococci were made on blood-agar slants. Small, discrete, biconvex, glistening colonies developed causing a distinct wide zone of hemolysis along the streak of inoculation.

¹¹ Jour. Med. Research, 1914, 31, p. 455.

The results of the cultures of the swabs and the crypts of the tonsils in 20 cases are indicated for comparison in detail in Table 1. These 20 are fairly representative of the entire group, and a glance at the table will indicate the great predominance of the hemolytic streptococci in the crypts.

TABLE 1
STREPTOCOCCI IN TONSILS

No.	Age	Sex	Pharynx		Right Tonsil Surface		Left Tonsil Surface		Right Tonsil Crypts		Left Tonsil Crypts	
			Hem-olytic Strep-to-cocci	Non-hem-olytic Strep-tococci	Hem-olytic Strep-to-cocci	Non-hem-olytic Strep-tococci	Hem-olytic Strep-to-cocci	Non-hem-olytic Strep-tococci	Hem-olytic Strep-to-cocci	Non-hem-olytic Strep-tococci	Hem-olytic Strep-to-cocci	Non-hem-olytic Strep-tococci
2	12	F	+	++	+	++	+	++	++++	+	++++	+
3	13	F	+	++++	+	++++	+	++++	++	+	++++	++++
4	15	F	+	++	+	++	+	++	+	++	+	++
5	14	F	+	++	+	++	+	++	++++	0	++++	0
6	6	F	0	++++	+	++	0	++++	++++	0	++++	0
7	7	F	+	++	+	++	+	++	++++	0	++++	0
8	13	M	0	++++	0	++++	0	++++	++++	0	++++	+
9Y	14	M	+	++++	+	++++	+	++++	++++	0	++++	0
10	7	F	+	++++	0	++++	+	++++	++	0	++	0
11	7	F	+	++++	+	++++	+	++++	++++	+	++++	0
12	6	F	+	++	+	++	+	++	++	+	++	+
13	10	M	+	++++	+	++++	+	+++	++++	+	++	++
14	13	M	+	++	+	++	+	++	++++	+	++++	+
15	12	F	+	++++	+	++++	+	++++	++	++	++	++
16	7	F	+	++++	+	++++	+	++++	++++	+	++++	0
17	16	M	0	++++	+	++++	0	++++	++	0	++	++
19	12	F	0	++++	+	++++	+	++++	++	+	++	+
20	12	M	+	++++	++	++	+	++++	++++	+	++	++
21	13	F	0	++++	+	++++	0	++++	++++	++++	++	++
22	12	M	+	++++	0	++++	0	++++	+	++++	++	++

TABLE 2
PERCENTAGE OF HEMOLYTIC STREPTOCOCCI IN SWABS AND CRYPT CULTURES OF 100 TONSILS

Swab cultures—	Per Cent.
Pharynx	43
Left tonsillar surface.....	49
Right tonsillar surface.....	59
One or both tonsillar surfaces.....	61
Cultures from crypts of excised tonsils—	
Right tonsil	92
Left tonsil	92
One or both tonsils.....	97

In Table 2 the frequency of hemolytic streptococci from the different sources is summarized. In cultures of the pharynx the colonies were few in number, constituting from 1-10% of the total. In no instance were they predominant. Similarly, in cultures from the tonsillar surfaces hemolytic streptococci were slightly more numerous, but still relatively few in number except in one instance where they were predominant. It is worthy of note that hemolytic streptococci were found on tonsillar surfaces in a decidedly higher percentage

(61%) than in the pharynx (43%). Of interest, too, is the occurrence of hemolytic streptococci in the crypts in 97% as compared with 61% on the tonsillar surfaces leaving 36 instances in which the streptococci were found in direct cultures of the crypts of excised tonsils from which surface swab cultures were negative. In all cases of positive swab cultures of the pharynx and tonsils, hemolytic streptococci were invariably found in larger numbers in the crypts. Another striking feature is the frequent predominance of hemolytic streptococci over all other micro-organisms in the crypts as indicated in Table 3.

TABLE 3
HEMOLYTIC STREPTOCOCCI IN THE CRYPTS OF EXCISED TONSILS

	Per Cent.
In Pure Culture—	
In both tonsils	11
In right or left.....	16
Predominating—	
In both tonsils.....	30
In right or left.....	17
In moderate number in both or in one tonsil.....	13
Few in both tonsils.....	10
None in both	3
None in one tonsil (present in the other).....	6

TABLE 4
ORGANISMS OTHER THAN STREPTOCOCCI FOUND IN AEROBIC CULTURES OF THROAT AND TONSILLAR CRYPTS

	Pharynx and Surface of Tonsil, %	Crypts of Excised Tonsils, %
Staphylococcus.....	40	30
Micrococcus catarrhalis.....	11	3
Pneumococcus.....	20	5
Diphtheroids.....	6	4
B. diphtheriae.....	1	0
Micrococcus tetragenus.....	1	0
B. mucosus.....	2	3

In Table 4 are listed the organisms encountered in the aerobic cultures. Pneumococci were differentiated from nonhemolytic streptococci by the appearance of typical colonies and by direct smear. No attempt was made at further differentiation by cultural and serologic studies since this would constitute a problem in itself. Nonhemolytic streptococci were found constantly as the predominating organism in 98% of pharyngeal cultures, and in 97% of swab cultures of tonsillar surfaces. In the crypts they were found in 73%, and here they were less numerous than in the surface cultures as is shown in Table 1. It is probable that the nonhemolytic streptococci would be more common

and numerous in the crypts if anaerobic cultures were also made, for we have demonstrated strongly anaerobic green-producing streptococci in connection with the actinomyces-like granules of the tonsils.⁹

In ascites-carbohydrate broth the hemolytic streptococci produce a flaky sediment with the small flocculi settling on the sides of the tube while the supernatant fluid with few exceptions remains clear. Rarely a slight turbidity is produced. Smears reveal gram-positive cocci usually in long chains. In most instances the cocci are spherical and only a few display a tendency to flattening of the opposing surfaces. Ninety strains were inoculated into the 4 differential sugar mediums—lactose, salicin, mannite and inulin. The medium was enriched with ascites fluid 1 part to 4 parts of 1% carbohydrate broth to which litmus solution was added as indicator. Readings were made at the end of 4 and 10 days. All strains fermented lactose, all but 5 fermented salicin, only 2 fermented mannite, and none inulin. According to Holman's¹² classification, they would fall chiefly in the *Streptococcus pyogenes* group; 1 strain would be classed as the *Streptococcus infrequens*, another as the *Streptococcus hemolyticus*.

TABLE 5
HYDROGEN-ION DETERMINATIONS IN BROTH CULTURES OF HEMOLYTIC STREPTOCOCCI*

Strains No.	P _H + Electrometric	P _H + Colorimetric
77.....	5.3	5.3
19.....	5.4	5.4
68.....	5.2	5.2
B.....	5.3	5.2
9Y.....	5.3	5.2
116.....	5.3	5.2
129.....	5.2	5.2
127.....	5.2	5.2
51.....	5.3	5.2
161.....	5.3	5.2
97.....	5.3	5.2
66.....	5.2	5.2
3.....	5.2	5.2
101.....	5.3	5.2
98.....	5.2	5.2

* We are indebted to Dr. Horry Jones for these determinations.

In litmus milk all strains produced acid. The clot formed spontaneously in five instances. Of the remainder, clotting was induced by gently heating to the boiling point for 1-5 minutes in all but 10 strains. This reaction in litmus milk is an important aid in differentiating the human strains from the far less pathogenic hemolytic streptococci of the lacticus group commonly found in raw milk. The milk streptococci, as shown by one of us,¹³ turns litmus tube rapidly white, coagulating the milk and forming a firm clot spontaneously. The strains from the tonsils correspond to the human type of hemolytic streptococci.

Further confirmation is furnished by the determination of the limiting hydrogen-ion concentration of the tonsil strains in carbohydrate broth cultures (1% lactose or dextrose ascitic broth cultures incubated 96 hours). The results are given in Table 5. The P_H values correspond to those of Ayres, Johnson and Davis¹⁴ who, employing dextrose yeast peptone medium, found a P_H value of 5.4 to 6.0 for pathogenic streptococci as compared with value of 4.6 to 4.7

¹² Jour. Med. Research, 1916, 34, p. 377.

¹³ Davis, D. J.: Jour. Infect. Dis., 1916, 19, p. 236.

¹⁴ Jour. Infect. Dis., 1918, 23, p. 290.

for the nonpathogenic streptococci. Smillie,⁷ using dextrose infusion broth, found a limiting hydrogen-ion concentration of 5.5 for 4 strains of hemolytic streptococci.

TABLE 6
STREPTOCOCCI IN NORMAL THROATS

Source	Number of Cases	Hemolytic Streptococci		Nonhemolytic Streptococci Percentage Positive
		Number Positive	Percentage Positive	
Normal Throats:				
Pharynx.....	24	6	25	100
Surface of tonsils.....	24	14	58	100
Gums.....	24	0	0	100
Nose.....	24	0	0	16
Streptococci in Throats of Individuals without Tonsils:				
Pharynx.....	19	3	15.8	100
Surface of tonsils.....	19	3	15.8	100
Gums.....	19	0	0	100
Nose.....	3	0	0	0

Twenty-two strains of hemolytic streptococci were selected at random and one blood-agar slant (incubated 48 hours) culture suspended in physiologic salt solution was injected intravenously in young rabbits. One animal died within 4 days, and four others within the 4th to the 10th day. The remaining 7 rabbits all had lesions and were killed on the 10th day. The rabbits lost from 10-130 gm. in weight rapidly. Within 48 hours several joints became enlarged. The lesions were chiefly a seropurulent or markedly purulent polyarthritis of the wrists, phalangeal joints, knees, ankles, occasionally the hip and shoulder joints. In one instance the right sternoclavicular joint was involved. Subpericardial, subpleural and subperitoneal hemorrhages occurred frequently. Verrucose endocarditis of the mitral valves was noted in three instances, of the tricuspid once, serofibrinous pericarditis and peritonitis once each. Tenosynovitis and hemorrhages into the skeletal muscles were occasionally encountered. In two instances (9 and 16) there appeared unilateral purulent iritis and iridocyclitis with pus in the anterior chamber from which hemolytic streptococci were recovered. The lesions correspond closely to those produced by streptococci isolated from the tear sac and tonsils by others.^{15, 16} In three animals three-fourths and one-half of one blood-agar slant were fatal. In another, one-fourth tube produced a slight joint lesion. In the fatal cases hemolytic streptococci identical with those injected were almost uniformly recovered from the joints and the heart blood. In rabbits surviving for 10 days the affected joints were usually sterile and the heart blood gave no growth.

The morphological and cultural characteristics of these hemolytic streptococci are identical with those of the hemolytic streptococci isolated from empyema, bronchopneumonia, and other fatal streptococcus infections.¹⁰ The uniformity of the strains in these respects together with their uniform virulence for rabbits points strongly to

¹⁵ Rosenow: Jour. Infect. Dis., 1915, 17, p. 402.

¹⁶ Irons, Brown and Nadler: Ibid., 1916, 18, p. 315.

the homogeneity of the human streptococcus hemolyticus group as recently determined by Kinsella and Smith¹⁷ by means of complement fixation tests.

Hemolytic streptococci, being found as we have shown in swab cultures in 61% and in the crypts of 97% of excised tonsils showing chiefly hyperplasia, the question arises as to the frequency of the same organisms in the crypts of the normal tonsil. It has been stated that in swab cultures of normal tonsils Ruediger⁶ found hemolytic streptococci in 59% and Smillie⁷ in 50%; and in cultures direct from crypts Nichols and Bryan¹⁰ found them in 50%. These figures are strikingly similar, and vary only 2-11% from the percentages with which hemolytic streptococci were isolated from the surface of hyperplastic tonsils. It is difficult to make cultures direct from the crypts with the tonsil in situ because of the almost unavoidable contamination with bacteria from the tonsillar surface and about the outlet of the crypt. This method therefore tends to increase the number of green-producing streptococci and other bacteria in the cultures and is not reliable.

In order to obtain further evidence whether the tonsils are a source of hemolytic streptococci normally, especially the crypts, a comparative study was made of the pharynx and tonsils of healthy individuals as compared with the throat of normal persons whose tonsils had been totally or partially removed some time previously. This comparison was made with a view to determine the possible influence of such removal on the occurrence of hemolytic streptococci in the throat.

Swab cultures were first made from the pharynx and the surface of each tonsil, from the gum margin and the nose, of 24 healthy persons who gave no history of recent sore throat and whose tonsils showed no gross evidence of acute or chronic inflammation. This group was composed of medical students, a few hospital interns, and several patients recently admitted to the surgical ward following trauma. They ranged in age from 20-30 years. Cultures were made in 10% blood agar by the poured plate method and the plates incubated at 37.5 C. for 48 hours. Readings were made at the end of 24 and 48 hours.

In cultures of the pharynx from this group hemolytic streptococci were found in 25%; in cultures of the surface of the tonsils in 58%. In all instances the number of colonies of hemolytic streptococci was less than 10% of the total colonies on the plate. In no instance were they found on the gum margin or in the nose. Nonhemolytic streptococci were noted in all the cultures from the pharynx and tonsils and were particularly numerous from the gums (Table 6).

¹⁷ Jour. Exper. Med., 1918, 28, p. 169.

In a second series similar cultures were made from 24 individuals of the same age (20-30) whose tonsils had been removed. In all but two the date of excision was from 6 months to 8 years previously; in two instances the tonsils had been removed 2 and 3 weeks previously, and the surfaces appeared congested and edematous. In 3 other persons remnants of tonsillar tissue appeared on either one or both sides. There are therefore nineteen instances in which no visible remnants of tonsils and no evidence of inflammation appeared. Cultures from these nineteen revealed hemolytic streptococci in scant numbers in three (15.8%) while nonhemolytic streptococci were present approximately in the same numbers as in the first series. In the two recently operated throats hemolytic streptococci were found in both. Two of the three individuals having remnants of tonsils gave hemolytic streptococci.

TABLE 7
INCIDENCE OF HEMOLYTIC STREPTOCOCCI IN THE THROAT AND SPUTUM IN VARIOUS
INFECTIOUS DISEASES

Disease	Investigators	Number of Cases	Percentage Giving Hemolytic Streptococci
Scarlet fever.....	Anthony.....	29	76
Scarlet fever.....	Ruediger.....	75	96
Scarlet fever.....	Wohl and Detweiler.....	27	100
Measles.....	Anthony.....	24	83.3
Measles.....	Ruediger.....	9	55.5
Measles.....	Cole and MacCallum.....	69	56.5
Measles.....	Simonin.....	45	60
Measles.....	Davis.....	23	100
Measles.....	Levy and Alexander.....	388	77.1
Influenza.....	Mathers.....	61	75.4
Influenza.....	Wohl and Detweiler.....	31	100
Influenza.....	Moody and Capps.....	30	100
Influenza.....	Nuzum, Pilot and others.....	100	37
Influenza.....	Hastings and Niles.....	39	28.6
Lobar pneumonia.....	Cole and MacCallum.....	26	57.7
Lobar pneumonia.....	Stone and others.....	59	50
Lobar pneumonia.....	Hastings and Niles.....	66	25.5

Comparing the two series it is seen that hemolytic streptococci were present in 15.8% of the throats of individuals without tonsils as compared with 58% of throats with normal tonsils. Excision of the tonsils therefore seems to reduce the frequency of hemolytic streptococci without any effect on the nonhemolytic; the results further indicate that the tonsils are the chief foci harboring hemolytic streptococci. Nichols and Bryan report the disappearance of hemolytic streptococci from the throat in 27 of 31 patients 11 days after extirpation of the diseased tonsils. Since remnants of tonsillar tissue may

harbor hemolytic streptococci it is possible in the three instances showing these organisms in the second series that the foci may have been in tonsil remnants not readily visible.

The high frequency of hemolytic streptococci in the human throat may explain the important rôle these organisms play as secondary invaders in a multitude of diseases. As is well known in acute infectious diseases like scarlet fever, measles, small-pox, etc., in most instances death is not due to the primary etiologic factor, but to secondary infections, especially the streptococcus. It is significant that in various acute infections characterized by an acute inflammation of the nasopharyngeal passages and by angina, the hemolytic streptococcus can be demonstrated in large numbers in cultures from the throat, and that the same organism is more often found in the fatal complicating lesions than any other organism. In table 7 are given the results of different investigators in which streptococci definitely of the hemolytic type were demonstrated in throats or sputum in various diseases.

Hektoen¹⁸ found long chain streptococci in the blood of 12 of 100 cases before death in mild as well as in severe cases. Dick and Henry¹⁹ isolated hemolytic streptococci from the blood, lymph glands, spleen and urine of fatal cases. Pearce²⁰ frequently recovered streptococci which could not be differentiated from ordinary *Streptococcus pyogenes*, and regarded these as the most common cause of the complications of bronchopneumonia, otitis media, and other general infections. Councilman²¹ and others noted numerous masses of streptococci on the surface and in the substance of the necrotic lesions of the mouth. *Streptococcus* was the most common organisms in the lungs, heart and other organs of fatal cases. Perkins and Pay²² likewise found the *Streptococcus pyogenes* in 95% of the fatal cases, and recovered the same organism from the blood in 1-4 days before death in 8 of 13 cases. The latter investigators showed further that there was a diminution of complement by demonstrating a decreased bacteriolytic action on *B. coli* and *B. typhosus* of the serum of affected patient. Thompson by the same method found a loss of complement especially in cases where streptococci were later isolated from fatal lesions. He believed that the specific contagion of variola caused a diminution of complement which predisposed to secondary local and general infections.

The *Streptococcus pyogenes* is commonly associated with the diphtheria bacillus in the lesions of diphtheria. Hilbert²³ believed that a symbiotic relationship existed in cultures inoculated with both organisms. Councilman, Mallory and Pearce,²⁴ in their extensive studies in diphtheria, found the

¹⁸ Jour. Am. Med. Assn., 1903, 40, p. 685.

¹⁹ Jour. Infect. Dis., 1914, 15, p. 85.

²⁰ Med. and Surg. Reports of Boston City Hosp., 1899, p. 39.

²¹ Studies on the Path. and Etiol. of Variola and Vaccinia, Boston, p. 67.

²² Jour. Med. Research, 1903, 10, p. 163.

²³ Quoted from Hiss and Zinsser, A Textbook of Bacteriology, 1918, p. 31.

²⁴ Jour. Boston Soc. Med. Sc., 1900, 5, p. 139.

Streptococcus pyogenes to be the most frequent organism associated with the diphtheria bacillus in the complicating lesions of the lung, middle ears, the accessory sinuses and the serous cavities, and found streptococci even more frequently than the diphtheria bacillus in the heart blood, liver, spleen and kidneys.

In measles (Table 7) hemolytic streptococci have been found with great frequency in the swab cultures of the pharynx and tonsils both in past and recent epidemics. The percentage varies from 55-100%, and the organisms are stated to occur often in predominating numbers. The complications of empyema and bronchopneumonia were apparently caused by the same organism as reported by Levy and Alexander,¹ Fox and Hamburger,³ Stone,²⁵ Clendening,²⁰ Beals and others,²⁷ Lorey,²⁸ Craig,²⁹ Cole and MacCallum.⁴ The general opinion of most of these observers is that the *Streptococcus hemolyticus* is carried by droplet infection from a carrier to susceptible measles patients. These organisms according to present bacteriologic methods are similar in cultural characteristics and biologic reactions to the streptococci isolated from normal and diseased tonsils. Two possibilities present themselves to explain the epidemic form of the streptococcus complications. Either the measles virus had attained unusual virulence, rendering the patient highly susceptible to the hemolytic streptococci already present in the crypts of the tonsil, or that a strain of hemolytic streptococcus may have been greatly enhanced in its invasive properties by passage through several hosts until new susceptible individuals succumb to streptococcus empyema and bronchopneumonia without a primary measles infection.²⁶ Tunnicliff³⁰ has shown that there is a diminution in the phagocytic activity of the leukocytes during the early stages of measles when a leukopenia exists. Hektoen³¹ states that "the frequency of streptococcus infection in this disease depends in some degree on the reduction in the powers to react against streptococci."

Acute respiratory infections with influenza-like symptoms have been reported in which hemolytic streptococci were found constantly in the throat and sputum often in predominating numbers (Table 8). Mathers,³² Wohl and Detweiler, Moody and Capps,³³ Müller and Seligmann³⁴ were inclined to believe that these epidemics were due primarily to the *Streptococcus hemolyticus*. In the pandemic of 1889-90 the micro-organisms most constantly found were the streptococcus and the pneumococcus. In 1911, Hastings and Niles³⁵ reported 39 cases, hemolytic streptococci predominating in the sputum of 11. In the present pandemic (1918) the streptococcus is no doubt playing an important rôle in causing complications. Nuzum and Pilot³⁶ isolated hemolytic streptococci from the lungs in 43%, and occasionally from the heart blood (8 of 36 necropsies) and the pleural fluid. It was obtained in pure culture in 3 of 15 positive cultures from puncture of the lungs during life. Friedlander and

²⁵ Arch. Int. Med., 1918, 22, p. 409.

²⁶ Am. Jour. Med. Sc., 1918, 156, p. 575.

²⁷ Jour. Infect. Dis., 1918, 23, p. 475.

²⁸ Ztschr. f. Hyg. u. Infektionskr., 1909, 63, p. 135.

²⁹ Jour. Am. Med. Assn., 1905, 44, p. 1187.

³⁰ Jour. Infect. Dis., 1918, 22, p. 462.

³¹ Jour. Am. Med. Assn., 1918, 71, p. 1201.

³² Jour. Infect. Dis., 1917, 21, p. 1.

³³ Jour. Am. Med. Assn., 1916, 66, p. 1696.

³⁴ Berl. klin. Wchnschr., 1911, 42, p. 1636.

³⁵ Jour. Am. Med. Assn., 1918, 71, p. 1562.

³⁶ Jour. Am. Med. Assn., 1918, 71, p. 1562.

others³⁷ noted it in the lungs in 46.7%, while Synnott and Clark³⁸ found it commonly in the sputum and in the lungs. From personal observations we have been surprised by the small number of hemolytic streptococci and certain other organisms of the throat in the very early stages of the disease. Indeed, hemolytic streptococci were found less often than in the normal throat (31%). In the later stages, however, the organism appeared more frequently and in larger numbers until in individuals with complications of pneumonia they were often found to be predominant in the throat and, less frequently, in the sputum. In the lungs they were usually associated with the pneumococcus or the influenza bacillus, but often appeared in pure culture in the heart blood and serous cavities suggesting a terminal streptococcus invasion of the blood stream.

In pneumonia of the lobar type hemolytic streptococci are commonly encountered in the throat and sputum in addition to the pneumococcus (Table 7). Clendening reports lobar pneumonia to be complicated by hemolytic streptococcus infection in 14% of 319 cases. In blood cultures immediately after death in lobar pneumonia streptococci have been occasionally demonstrated. (Richey and Goehring.³⁹) In lobar pneumonia as in measles the question has arisen in connection with the epidemic form of streptococcus infections whether the complications are due most often to the streptococci already present in the tonsils of the patient; or, when epidemics are prevalent, to streptococci of enhanced virulence transmitted from patient to patient.

In other infectious diseases hemolytic streptococci have been frequently found. They were noted by Davis⁴⁰ in the throat of 57 of 61 cases of whooping cough, and in 8 of 11 cases of varicella.

In long standing diseases in which the state of resistance becomes progressively lowered the *Streptococcus pyogenes* is the most frequent cause of terminal infection. It is common to find at necropsy terminal infection with no distinct focal lesions. In his study on terminal infections, Flexner⁴¹ commonly encountered streptococci and mentions the tonsil as the possible atrium of infection in some cases. Gwyn and Harris⁴² recognized the streptococcus pyogenes in antemortem blood cultures as agonal invaders in cases of chronic parenchymatous nephritis, cirrhosis of the liver, leukemia, endocarditis and pneumonia. In cultures postmortem these organisms were isolated in the corresponding cases, and also in two cases of typhoid fever and in a case of ulceration of the larynx and esophagus which had given negative cultures antemortem. Strauch,⁴³ in 2,000 blood cultures postmortem, found the *Streptococcus pyogenes* altogether in 548; in 460 of this number it occurred alone. His series included cases of the acute exanthemata, chronic wasting diseases, and various forms of acute and chronic tuberculosis. In cultures taken immediately after death Fredette⁴⁴ encountered the same organism in 42 of 119 individuals with such diseases as leukemia, chronic endocarditis, pneumonia, appendicitis and others. Richey and Goehring³⁹ in a similar study recovered the organism in 14 of 19 positive cultures from 55 cases of chronic wasting diseases. Flexner showed that the bactericidal effect of serum from patients affected with chronic renal or cardiorenal diseases was less against *Staphylococcus aureus* than that

³⁷ Jour. Am. Med. Assn., 1918, 71, p. 1652.

³⁸ Ibid., 1918, 71, p. 1816.

³⁹ Jour. Med. Research, 1918, 38, p. 420.

⁴⁰ Jour. Infect. Dis., 1906, 3, p. 1.

⁴¹ Jour. Exper. Med., 1896, 1, p. 558.

⁴² Jour. Infect. Dis., 1902, 2, p. 514.

⁴³ Ztschr. f. Hyg. u. Infektionskr., 1910, 65, p. 183.

⁴⁴ Jour. Lab. and Clin. Med., 1916, 2, p. 180.

or normal serum. Later Longcope⁴⁵ examined the serum of 17 similar patients and observed a reduced bactericidal action on *B. coli* and *B. typhosus*, especially in those who subsequently succumbed to terminal infections. It would seem that the diminution of protective substances in the blood is to a large extent the underlying basis for the occurrence of terminal infections.

The regularity with which hemolytic streptococci can be demonstrated in the throats of patients affected with acute exanthemata, acute respiratory infections, diphtheria, pneumonia, etc., suggests that the common source of these organisms is from some preexisting focus which harbors these bacteria. It seems unlikely that the same organism could be so regularly introduced from without in such a variety of primary diseases. Streptococci of enhanced virulence may occasionally be transmitted in epidemic form by direct or indirect contact, by droplet infection and by milk, as in septic sort throat; but these routes are probably relatively unusual, although at times no doubt they exist and may prove most serious.

Three possible factors may be concerned in the production of secondary streptococcus infections. (1) The existing dormant organisms in the tonsil or throat may become enhanced in their virulence through symbiosis or in some unknown way by the primary virus (measles, scarlet fever, smallpox), or by the bacteria such as *B. diphtheriae*, pneumococcus or influenza bacillus. (2) They may become active through the diminution of the resistance of the local tissues of the respiratory passages. (3) They may attain increased invasive powers through the lowering of the general resistance of the host overwhelmed by an acute or chronic toxemia. Probably all three factors are combined in certain diseases, though one factor often appears to be more important than the others. Thus, in acute respiratory and throat infections, streptococci seem to spread from the tonsillar crypts to the adjacent mucous surfaces, descend into the bronchi and lungs, and enter the blood stream from these tissues. In chronic diseases, on the other hand, the diminution of the resisting power of the blood may admit the streptococci directly into the circulating blood stream without any marked local changes in the throat.

CONCLUSIONS

Hemolytic streptococci are common in the normal and diseased throat. The chief foci of these organisms are the crypts of the tonsils.

⁴⁵ Univ. of Penn. Med. Bull., 1902, 15, p. 331.

Hemolytic streptococci were recovered by swab cultures in sparse numbers from the pharynx and tonsillar surfaces in 61% of throats, chiefly children with hyperplastic tonsils; and from the crypts of the excised tonsils of the same individuals in 97%, usually in predominating numbers. Swab cultures are therefore unreliable in determining the incidence of this organism in the respiratory tract.

The frequency of hemolytic streptococci is decidedly less in the throats of persons whose tonsils have been extirpated than in the throats of persons with normal tonsils.

These streptococci agree in the main in their morphology, cultural characteristics, fermentation reactions and pathogenicity for rabbits, and are practically identical with hemolytic streptococci isolated from various human pathologic sources.

Hemolytic streptococci from the crypts of the tonsils are probably the most important source of the streptococcus complications of the various acute infectious diseases, and of the terminal infections.

A DOUBLE SUGAR MEDIUM FOR THE CULTURAL DIAGNOSIS OF INTESTINAL AND OTHER BACTERIA

ARTHUR I. KENDALL AND MARJORIE RYAN

From the Patten Research Laboratory, Northwestern University Medical School, Chicago.

The interest and importance which is associated with the study of intestinal infections and intestinal bacteria has led to a careful survey of the distribution of intestinal microbes in the feces in health and disease. One of the striking results of the exploration of this field is the demonstration of bacillus carriers — apparently normal individuals who harbor and excrete, regularly or intermittently, pathogenic organisms.

The recognition of bacillus carriers is quite as important from the point of view of public health as the identification of bacteria in frank acute infections. The list of organisms which incite specific disease and which may occur in the intestinal or urinary tracts of bacillus carriers is a formidable one, including the cholera, typhoid, bacillary dysentery and paratyphoid groups of organisms. Probably *Bacillus alcaligenes* and the Morgan bacillus should be regarded as occasional causative agents in this type of disease as well. The problem confronting the diagnostic laboratory is the isolation and identification of members of this intestinal group from material containing large numbers of normal intestinal types of organisms.

The identification of typhoid and paratyphoid bacilli in the urine of suspected carriers is relatively simple, although *Bacillus coli* and *Bacillus proteus*, frequent incitants of cystitis, are not infrequently present in large numbers at the same time.

Material submitted for the identification of pathogenic intestinal bacteria may offer no definite clue as to the organisms present and the problem clearly resolves itself into the identification or exclusion of all organisms that may reasonably be sought for. When considerable numbers of samples are studied at the same time, the amount of labor involved is great, and any procedure which will lessen the time and work, consistent with accuracy, is highly desirable. The method

commonly used is to isolate colonies from Endo plates and establish their identity by agglutination with specific serum. Where small numbers of organisms are involved, this is very rapid and satisfactory. The entire process, using the rapid method of Kendall and Day,¹ may require less than 24 hours. This method is impracticable when large numbers of organisms await identification, however, and an additional weeding out of nonspecific organisms is necessary before agglutination is practiced.

So-called "slow" strains of *B. coli*, which fail to ferment the lactose of Endo plates, certain strains of streptococci and *B. proteus*, are among the organisms to be eliminated before the agglutination test is made. "Slow" colon bacilli usually ferment lactose when they are transferred to suitable mediums from the Endo plate, and many strains of streptococci which produce colorless colonies on this medium ferment readily on a retransfer to lactose or saccharose mediums. The employment of suitable diagnostic mediums as an adjunct to the Endo plate, provided the time element is not disproportionately increased, therefore, is clearly advantageous.

Russell² made a noteworthy contribution to the subject by the introduction of the double sugar medium. The Russell medium contains the usual protein ingredients of nutrient agar, and, in addition, 0.1% dextrose and 1% lactose. Litmus is added as an indicator and the medium is slanted before inoculation. The culture is smeared on the surface, and implanted deep into the butt of the tube with the needle.

Organisms, as *B. alcaligenes* which ferments no sugars, yield a blue or blue-violet uniform growth. Those, as typhoid and dysentery bacilli, which ferment glucose but not lactose, develop acidity in the depths of the medium leaving the surface unchanged in color, or slightly more alkaline. Bacteria which form gas from glucose but not from lactose produce gas bubbles in the depths, leaving the upper surface of the slant practically unchanged in color and appearance. Lactose fermenters color the entire medium red, and, if they produce gas, fill it with gas bubbles as well.

Since the Russell medium was published, new double and triple sugar mediums have appeared,^{3, 4} which have added materially to the

¹ Jour. Med. Research, 1911, 20, p. 95.

² Jour. Med. Research, 1911, 25, p. 217.

³ Kligler and Defandorfer: Jour. Bacteriol., 1918, 3, p. 437.

⁴ Krumwiede and Kohn: Jour. Med. Research, 1917, 37, p. 225.

rapidity of cultural diagnosis of special organisms. The use of the Andrade indicator,⁵ which is fuchsin decolorized by NaOH, has been found much more satisfactory than litmus as an indicator of acid formation by bacteria. Obviously, the Endo indicator would be practically as efficient except for the fact that it is more sensitive to light. For this reason the Andrade indicator is advantageously substituted for the original Endo indicator in the Endo medium.

The carbohydrates of importance in the cultural identification of intestinal organisms are glucose, lactose and saccharose, and the alcohol mannitol. Table 1, which summarizes the reactions of the more important pathogenic organisms and those of the normal intestinal tract likely to be confused with them, shows this schematically:

TABLE 1
PATHOGENIC AND NONPATHOGENIC ORGANISMS COMMONLY FOUND IN THE INTESTINAL TRACT

Name	Glucose	Lactose	Saccharose	Mannitol
<i>B. Alcaligenes</i> *.....	—	—	—	—
<i>B. dysenteriae</i> Shiga.....	+	—	—	—
<i>B. dysenteriae</i> Flexner.....	+	—	—	+
<i>B. dysenteriae</i> Strong.....	+	—	+	+
<i>B. typhosus</i>	+	—	—	+
<i>B. No. 1</i> Morgan.....	g	—	—	—
<i>B. paratyphosus</i> A.....	g	—	—	g
<i>B. paratyphosus</i> B.....	g	—	—	g
<i>B. coli</i> A.....	g	g	—	g
<i>B. coli</i> B.....	g	g	g	g
<i>B. proteus</i>	g	—	g	—
<i>Vib. cholera</i>	+	+	+	+
<i>Streptococcus</i>	+	+	+	±

* Explanation: — = alkaline reaction, no fermentation; + = acid reaction, fermentation without gas; g = acid reaction, gaseous fermentation.

The Russell medium distinguishes clearly between the glucose-acid, glucose-gas, and lactose-acid and lactose-gas producing strains. The utilization of another double sugar containing saccharose 1%, and mannitol 0.1%, while not adding materially to the amount of work involved, distinguishes between the various saccharose acid and gas, and the mannitol acid and gas producers, thus materially increasing the definiteness of cultural diagnosis and reducing somewhat the necessity for final agglutination with specific or polyvalent serum.

The reactions of the various organisms on the double sugars are given in Table 2.

⁵ Abstract of Sanitary Reports, Pub. Health and Mar. Hosp. Ser., 1895, 10, p. 679.

Preparation of Saccharose—Mannitol Medium.—Nutrient agar, containing 2.5% agar, is prepared in the usual manner, and the reaction is adjusted to such a degree that the color, when the Andrade indicator is added, is faintly pink when hot. It is extremely important to weigh the mannitol exactly.

*Andrade Indicator.**—A half of 1% aqueous solution of acid fuchsin is carefully treated with 10% caustic soda until the color is just discharged. The solution may be sterilized in the Arnold or in the autoclave. One per cent. of this solution is added to the medium, which has been previously titrated to the neutral point of this indicator. The Andrade indicator may be sterilized with the medium without impairing its tinctorial value or injuring the nutritive properties of the medium. The medium is slanted in the usual manner, using a sufficient quantity to make the butt at the point where the slanted surface begins about 1 cm. in depth. The medium so prepared is colorless or very faintly pink when cold.

TABLE 2
REACTIONS OF THE VARIOUS ORGANISMS ON DOUBLE SUGARS

Name	0.1% Glucose 1% Lactose		0.1% Mannitol 1% Saccharose	
	Butt	Slant	Butt	Slant
<i>B. alcaligenes</i>	c	c	c	c
<i>B. dysenteriae</i> Shiga.....	r	c	c	c
<i>B. dysenteriae</i> Flexner.....	r	c	r	c
<i>B. dysenteriae</i> Strong.....	r	c	r	r
<i>B. typhosus</i>	r	c	r	c
<i>B. No. 1</i> Morgan.....	g	c	c	c
<i>B. paratyphosus</i> A.....	g	c	g	c
<i>B. paratyphosus</i> B.....	g	c	g	c
<i>B. coli</i> A.....	g	r	g	c
<i>B. coli</i> B.....	g	r	g	r
<i>B. proteus</i>	g	c	g	r
<i>B. cholerae</i>	r	r	r	r
<i>Streptococcus</i>	r	r	r	r

Explanation: c = colorless (no fermentation); r = acid fermentation, red color in butt of tube or on slanted surface; g = red and gas bubbles, gaseous fermentation.

Inoculation is practised both on the slanted surface and in the depth of the medium with a rather long platinum needle.

This saccharose-mannitol medium and the Russell glucose-lactose medium together give the fermentation reactions of the pathogenic and nonpathogenic intestinal bacilli in the four carbohydrate mediums of diagnostic importance for this group, thus reducing the amount of time and material required one-half. The indications resulting from the fermentation reactions lead directly to the agglutination of the organisms with specific or polyvalent serums in the usual manner for confirmation. The condensation water of the glucose-lactose medium is favorable for this purpose.

* Andrade (Jour. Med. Research, 1906, 14, p. 551) states that 0.0001 gm. of KOH unites with 0.005 gm. of Grüber's acid fuchsin. The indicator is very sensitive to acid. 0.00003 gm. responds to 0.001 gm. pure HCl by the production of a distinct red color.

SUMMARY

A new double sugar medium is described which affords a rapid, accurate and convenient means for determining the fermentation reactions of bacteria simultaneously in saccharose and mannitol.

The mannitol must be weighed accurately. An excess above 0.1% of this alcohol will lead to the development of an excessive acid reaction, with mannitol fermenting organisms, which will permanently color the entire surface of the slanted area.

The reaction of the medium must be carefully adjusted to the point of extinction of color of the acid fuchsin.

The Russell double sugar medium and the medium described here will give all the essential fermentation reactions of the important intestinal bacteria of pathogenic importance.

Confirmatory agglutination with appropriate serum can be readily carried out, using the culture in the condensation water of the Russell glucose-lactose tube, as the source of the culture.

The double sugar mediums can be advantageously applied to the cultural diagnosis of aerobic bacteria in general.

FURTHER OBSERVATIONS ON THE INFLUENCE OF INCUBATION ON THE WASSERMANN REACTION

E. H. RUEDIGER

From the Pathological Laboratory of the Bismarck Hospital, Bismarck, N. D.

In a previous report¹ it was shown that complement was bound better at a temperature of about 10 C. than at 37 C. It was shown that complement binding progressed slowly so that many hours are required for its completion. At that time the optimum temperature for complement binding and the time necessary for its completion had not been determined and will be dealt with at this time.

In the present report the following points are briefly considered.

1. Incubation at 15 C., 5 C., and 2 C. for 5 hours, 10 hours and 15 hours compared with incubation at 10 C. for 5 hours, 10 hours and 15 hours.

2. Incubation at 1 C., 2 C., 3 C., 4 C., 6 C., 7 C., 8 C. and 9 C. for 5 hours and 10 hours compared with incubation at 5 C. for 5 hours and 10 hours.

3. Incubation at 3 C., 1 C. and 0.5 C. for 5 hours and for 10 hours compared with incubation at 2 C. for 5 hours and for 10 hours.

4. Incubation at 0.5 C. for 5 hours and for 10 hours compared with incubation at 1 C. for 5 hours and for 10 hours.

5. Fifteen-hour incubation at 1 C. compared with 24-hour incubation at 1 C.

6. Fifteen-hour incubation at 1 C. compared with 10-hour incubation at 1 C. Normal serum.

7. Acetone insoluble antigen compared with alcoholic extract, incubation at 1 C. and 37 C. for 1 hour and for 10 hours.

8. Alcoholic extract of beef heart compared with alcoholic extract of human heart, incubation at 1 C. and 37 C. for 1 hour and for 10 hours.

9. Acetone insoluble antigen of beef heart compared with alcoholic extract of human heart, incubation at 1 C. and at 37 C. for 1 hour and for 10 hours.

10. Nine and one-half hour incubation at 1 C. + one-half hour incubation at 37 C. compared with 10-hour incubation at 1 C.

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¹ Jour. Infect. Dis., 1918, 23, p. 173.

TEST 1

Serums 1 to 18, inclusive, incubation at 15 C., 5 C. and 2 C. for 5 hours, 10 hours and 15 hours compared with incubation at 10 C. for 5 hours, 10 hours and 15 hours. The technic of all tests in this report was identical with the technic described in the previous reports.

TABLE 1

INCUBATION AT 15 C., 5 C. AND 2 C. COMPARED WITH INCUBATION AT 10 C.

Number of Serum	Incubation Tempera- ture, C.	Incubation Time, Hours	Readings*						Results
			Antigen Tubes			Control Tubes			
			1	2	3	1'	2'	3'	
1	10	5	+	+	0	+	+	±	Moderately positive, 2+
	15	5	+	+	0	+	+	±	Moderately positive, 2+
	10	10	+	±	0	+	+	±	Strongly positive, 3+
	15	10	+	+	0	+	+	±	Moderately positive, 2+
	10	15	+	±	0	+	+	±	Strongly positive, 3+
	15	15	+	±	0	+	+	±	Strongly positive, 3+
3	10	5	+	tr	0	+	+	±	Strongly positive, 4+
	15	5	+	±	0	+	+	±	Strongly positive, 3+
	10	10	+	tr	0	+	+	±	Strongly positive, 4+
	15	10	+	±	0	+	+	±	Strongly positive, 3+
	10	15	+	0	0	+	+	±	Strongly positive, 5+
	15	15	+	±	0	+	+	±	Strongly positive, 3+
3	10	5	+	±	0	+	+	±	Strongly positive, 3+
	15	5	+	+	0	+	+	±	Moderately positive, 2+
	10	10	+	tr	0	+	+	±	Strongly positive, 4+
	15	10	+	±	0	+	+	±	Strongly positive, 3+
	10	15	+	0	0	+	+	±	Strongly positive, 5+
	15	15	+	±	0	+	+	±	Strongly positive, 3+
4	10	5	+	0	0	+	+	±	Strongly positive, 5+
	15	5	+	tr	0	+	+	±	Strongly positive, 4+
	10	10	±	0	0	+	+	±	Strongly positive, 6+
	15	10	+	tr	0	+	+	±	Strongly positive, 4+
	10	15	tr	0	0	+	+	±	Strongly positive, 8+
	15	15	+	0	0	+	+	±	Strongly positive, 5+
5	10	5	+	tr	0	+	+	±	Strongly positive, 4+
	15	5	+	+	0	+	+	±	Moderately positive, 2+
	10	10	+	0	0	+	+	±	Strongly positive, 5+
	15	10	+	±	0	+	+	±	Strongly positive, 3+
	10	15	+	0	0	+	+	±	Strongly positive, 5+
	15	15	+	±	0	+	+	±	Strongly positive, 3+
6	10	5	+	0	0	+	+	±	Strongly positive, 5+
	15	5	+	+	0	+	+	±	Moderately positive, 2+
	10	10	±	0	0	+	+	±	Strongly positive, 6+
	15	10	+	±	0	+	+	±	Strongly positive, 3+
	10	15	±	0	0	+	+	±	Strongly positive, 6+
	15	15	+	tr	0	+	+	±	Strongly positive, 4+
7	10	5	±	0	0	+	+	±	Strongly positive, 6+
	5	5	±?	0	0	+	+	±	Strongly positive, 7+
	10	10	±	0	0	+	+	±	Strongly positive, 6+
	5	10	0	0	0	+	+	±	Strongly positive, 10+
	10	15	±	0	0	+	+	±	Strongly positive, 6+
	5	15	0	0	0	+	+	±	Strongly positive, 10+
8	10	5	+	0	0	+	+	±	Strongly positive, 5+
	5	5	±	0	0	+	+	±	Strongly positive, 6+
	10	10	+	0	0	+	+	±	Strongly positive, 5+
	5	10	tr	0	0	+	+	±	Strongly positive, 8+
	10	15	+	0	0	+	+	±	Strongly positive, 5+
	5	15	0	0	0	+	+	tr	Strongly positive, 8+

* Explanation: In all tables + means complete hemolysis; ±, hemolysis between 50% and 100%; tr (trace), hemolysis up to 50%; 0, no hemolysis.

TABLE 1—Continued

INCUBATION AT 15 C., 5 C. AND 2 C. COMPARED WITH INCUBATION AT 10 C.

Number of Serum	Incubation Temperature, C.	Incubation Time, Hours	Readings						Results	
			Antigen Tubes			Control Tubes				
			1	2	3	1'	2'	3'		
9	10	5	±	0	0	+	+	±	Strongly positive,	6+
	5	5	±?	0	0	+	+	±	Strongly positive,	7+
	10	10	±	0	0	+	+	±	Strongly positive,	6+
	5	10	0	0	0	+	+	±	Strongly positive,	10+
	10	15	±	0	0	+	+	±	Strongly positive,	6+
	5	15	0	0	0	+	+	±	Strongly positive,	10+
10	10	5	+	tr	0	+	+	±	Strongly positive,	4+
	5	5	+	tr	0	+	+	±	Strongly positive,	4+
	10	10	+	0	0	+	+	±	Strongly positive,	5+
	5	10	±	0	0	+	+	±	Strongly positive,	6+
	10	15	+	0	0	+	+	±	Strongly positive,	6+
	5	15	tr	0	0	+	+	±	Strongly positive,	8+
11	10	5	+	0	0	+	+	±	Strongly positive,	5+
	5	5	+	0	0	+	+	±	Strongly positive,	5+
	10	10	+	0	0	+	+	±	Strongly positive,	5+
	5	10	±	0	0	+	+	±	Strongly positive,	6+
	10	15	+	0	0	+	+	±	Strongly positive,	5+
	5	15	tr	0	0	+	+	tr	Strongly positive,	6+
12	10	5	+	tr	0	+	+	±	Strongly positive,	4+
	5	5	+	0	0	+	+	±	Strongly positive,	5+
	10	10	+	tr	0	+	+	±	Strongly positive,	4+
	5	10	±	0	0	+	+	±	Strongly positive,	6+
	10	15	+	0	0	+	+	tr	Strongly positive,	4+
	5	15	tr	0	0	+	+	tr	Strongly positive,	6+
13	10	5	+	±	0	+	+	±	Strongly positive,	3+
	2	5	+	±	0	+	+	±	Strongly positive,	3+
	10	10	+	tr	0	+	+	±	Strongly positive,	4+
	2	10	+	tr	0	+	+	±	Strongly positive,	4+
	10	15	+	tr	0	+	+	±	Strongly positive,	4+
	2	15	+	tr	0	+	+	±	Strongly positive,	4+
14	10	5	+	tr	0	+	+	±	Strongly positive,	4+
	2	5	±	0	0	+	+	±	Strongly positive,	6+
	10	10	+	tr	0	+	+	±	Strongly positive,	4+
	2	10	tr	0	0	+	+	±	Strongly positive,	8+
	10	15	+	tr	0	+	+	±	Strongly positive,	4+
	2	15	tr	0	0	+	+	±	Strongly positive,	8+
15	10	5	+	tr	0	+	+	±	Strongly positive,	4+
	2	5	+	0	0	+	+	±	Strongly positive,	5+
	10	10	+	tr	0	+	+	±	Strongly positive,	4+
	2	10	+	0	0	+	+	±	Strongly positive,	5+
	10	15	+	tr	0	+	+	±	Strongly positive,	4+
	2	15	±	0	0	+	+	±	Strongly positive,	6+
16	10	5	±	0	0	+	+	±	Strongly positive,	6+
	2	5	tr	0	0	+	+	±	Strongly positive,	8+
	10	10	±	0	0	+	+	±	Strongly positive,	6+
	2	10	0	0	0	+	+	±	Strongly positive,	10+
	10	15	±	0	0	+	+	±	Strongly positive,	6+
	2	15	0	0	0	+	+	±	Strongly positive,	10+
17	10	5	+	tr	0	+	+	±	Strongly positive,	4+
	2	5	+	0	0	+	+	±	Strongly positive,	5+
	10	10	+	0	0	+	+	±	Strongly positive,	5+
	2	10	±	0	0	+	+	±	Strongly positive,	6+
	10	15	+	0	0	+	+	±	Strongly positive,	5+
	2	15	±	0	0	+	+	±	Strongly positive,	6+
18	10	5	+	0	0	+	+	±	Strongly positive,	5+
	2	5	tr	0	0	+	+	±	Strongly positive,	8+
	10	10	±	0	0	+	+	±	Strongly positive,	6+
	2	10	0	0	0	+	+	±	Strongly positive,	10+
	10	15	±	0	0	+	+	±	Strongly positive,	6+
	2	15	0	0	0	+	+	±	Strongly positive,	10+

Table 1 shows the results obtained by comparing incubation at 15 C., at 5 C. and at 2 C. for 5 hours, for 10 hours and for 15 hours with incubation at 10 C. for 5, 10 and 15 hours. Incubation at 10 C. gave stronger positive results than did incubation at 15 C., and incubation at 5 C. and at 2 C. gave stronger positive results than did incubation at 10 C. Incubation for 10 hours gave stronger positive results than did incubation for 5 hours, and incubation for 15 hours gave stronger positive results than did incubation for 10 hours.

TEST 2

Serums 19 to 66, incubation at 1 C., 2 C., 3 C., 4 C., 6 C., 7 C., 8 C. and 9 C. for 5 hours and 10 hours was compared with incubation at 5 C. for 5 hours and 10 hours.

TABLE 2
INCUBATION AT 1 C., 2 C., 3 C., 4 C., 6 C., 7 C., 8 C. AND 9 C. COMPARED WITH
INCUBATION AT 5 C.

Number of Serum	Incubation Tempera- ture, C.	Incubation Time, Hours	Readings						Results	
			Antigen Tubes			Control Tubes				
			1	2	3	1'	2'	3'		
19	5	5	+	+	0	+	+	±	Moderately positive,	2+
	1	5	+	+	0	+	+	±	Moderately positive,	2+
	5	10	+	+	0	+	+	±	Moderately positive,	2+
	1	10	+	±	0	+	+	±	Strongly positive,	3+
20	5	5	+	±	0	+	+	±	Strongly positive,	3+
	1	5	+	tr	0	+	+	±	Strongly positive,	4+
	5	10	+	tr	0	+	+	±	Strongly positive,	4+
	1	10	+	0	0	+	+	±	Strongly positive,	5+
21	5	5	+	tr	0	+	+	±	Strongly positive,	4+
	1	5	tr	0	0	+	+	±	Strongly positive,	8+
	5	10	tr	0	0	+	+	±	Strongly positive,	8+
	1	10	0	0	0	+	+	±	Strongly positive,	10+
22	5	5	+	0	0	+	+	±	Strongly positive,	5+
	1	5	tr	0	0	+	+	±	Strongly positive,	8+
	5	10	tr	0	0	+	+	±	Strongly positive,	8+
	1	10	0	0	0	+	+	±	Strongly positive,	10+
23	5	5	+	±	0	+	+	±	Strongly positive,	3+
	1	5	+	tr	0	+	+	±	Strongly positive,	4+
	5	10	+	0	0	+	+	tr	Strongly positive,	4+
	1	10	tr	0	0	+	+	tr	Strongly positive,	6+
24	5	5	+	tr	0	+	+	±	Strongly positive,	4+
	1	5	+	0	0	+	+	±	Strongly positive,	5+
	5	10	+	0	0	+	+	±	Strongly positive,	5+
	1	10	0	0	0	+	+	±	Strongly positive,	10+
25	5	5	+	±	0	+	+	±	Strongly positive,	3+
	2	5	+	±	0	+	+	±	Strongly positive,	3+
	5	10	+	tr	0	+	+	±	Strongly positive,	4+
	2	10	+	tr	0	+	+	±	Strongly positive,	4+
26	5	5	+	0	0	+	+	±	Strongly positive,	5+
	2	5	tr	0	0	+	+	±	Strongly positive,	8+
	5	10	tr	0	0	+	+	±	Strongly positive,	8+
	2	10	0	0	0	+	+	±	Strongly positive,	10+

TABLE 2—Continued

INCUBATION AT 1 C., 2 C., 3 C., 4 C., 6 C., 7 C., 8 C. AND 9 C. COMPARED WITH
INCUBATION AT 5 C.

Number of Serum	Incubation Tempera- ture, C.	Incubation Time, Hours	Readings						Results	
			Antigen Tubes			Control Tubes				
			1	2	3	1'	2'	3'		
27	5	5	tr	0	0	+	+	±	Strongly positive,	8+
	2	5	0	0	0	+	+	±	Strongly positive,	10+
	5	10	0	0	0	+	+	±	Strongly positive,	10+
	2	10	0	0	0	+	+	±	Strongly positive,	10+
28	5	5	+	0	0	+	+	±	Strongly positive,	5+
	2	5	+	0	0	+	+	±	Strongly positive,	5+
	5	10	±	0	0	+	+	±	Strongly positive,	6+
	2	10	tr	0	0	+	+	±	Strongly positive,	8+
29	5	5	tr	0	0	+	+	tr	Strongly positive,	6+
	2	5	tr	0	0	+	+	±	Strongly positive,	8+
	5	10	0	0	0	+	+	tr	Strongly positive,	8+
	2	10	0	0	0	+	+	±	Strongly positive,	10+
30	5	5	tr	0	0	+	+	±	Strongly positive,	8+
	2	5	0	0	0	+	+	±	Strongly positive,	10+
	5	10	0	0	0	+	+	±	Strongly positive,	10+
	2	10	0	0	0	+	+	±	Strongly positive,	10+
31	5	5	tr	0	0	+	+	±	Strongly positive,	8+
	3	5	0	0	0	+	+	±	Strongly positive,	10+
	5	10	0	0	0	+	+	±	Strongly positive,	10+
	3	10	0	0	0	+	+	±	Strongly positive,	10+
32	5	5	+	tr	0	+	+	±	Strongly positive,	4+
	3	5	+	0	0	+	+	±	Strongly positive,	5+
	5	10	±	0	0	+	+	±	Strongly positive,	6+
	3	10	tr	0	0	+	+	±	Strongly positive,	8+
33	5	5	tr	0	0	+	+	±	Strongly positive,	8+
	3	5	0	0	0	+	+	±	Strongly positive,	10+
	5	10	0	0	0	+	+	±	Strongly positive,	10+
	3	10	0	0	0	+	+	±	Strongly positive,	10+
34	5	5	±	0	0	+	+	±	Strongly positive,	6+
	3	5	tr	0	0	+	+	±	Strongly positive,	8+
	5	10	tr	0	0	+	+	±	Strongly positive,	8+
	3	10	tr	0	0	+	+	±	Strongly positive,	8+
35	5	5	+	+	0	+	+	±	Moderately positive,	2+
	3	5	+	+	0	+	+	±	Moderately positive,	2+
	5	10	+	±	0	+	+	±	Strongly positive,	3+
	3	10	+	±	0	+	+	±	Strongly positive,	3+
36	5	5	+	+	0	+	+	±	Moderately positive,	2+
	3	5	+	+	0	+	+	±	Moderately positive,	2+
	5	10	+	±	0	+	+	±	Strongly positive,	3+
	3	10	+	tr	0	+	+	±	Strongly positive,	4+
37	5	5	+	+	tr	+	+	±	Weakly positive,	1+
	4	5	+	+	tr	+	+	±	Weakly positive,	1+
	5	10	+	+	0	+	+	±	Moderately positive,	2+
	4	10	+	+	0	+	+	±	Moderately positive,	2+
38	5	5	+	+	0	+	+	±	Moderately positive,	2+
	4	5	+	+	0	+	+	±	Moderately positive,	2+
	5	10	+	±	0	+	+	±	Strongly positive,	3+
	4	10	+	±	0	+	+	±	Strongly positive,	3+
39	5	5	+	+	0	+	+	±	Moderately positive,	2+
	4	5	+	+	0	+	+	±	Moderately positive,	2+
	5	10	+	±	0	+	+	±	Strongly positive,	3+
	4	10	+	tr	0	+	+	±	Strongly positive,	4+
40	5	5	+	tr	0	+	+	±	Strongly positive,	4+
	4	5	+	tr	0	+	+	±	Strongly positive,	4+
	5	10	+	0	0	+	+	±	Strongly positive,	5+
	4	10	+	0	0	+	+	±	Strongly positive,	5+

TABLE 2—Continued

INCUBATION AT 1 C., 2 C., 3 C., 4 C., 6 C., 7 C., 8 C. AND 9 C. COMPARED WITH
INCUBATION AT 5 C.

Number of Serum	Incubation Tempera- ture, C.	Incubation Time, Hours	Readings						Results
			Antigen Tubes			Control Tubes			
			1	2	3	1'	2'	3'	
41	5	5	+	+	0	+	+	±	Moderately positive, 2+
	4	5	+	+	0	+	+	±	Moderately positive, 2+
	5	10	+	+	0	+	+	±	Moderately positive, 2+
	4	10	+	±	0	+	+	±	Strongly positive, 3+
42	5	5	+	+	0	+	+	±	Moderately positive, 2+
	4	5	+	±	0	+	+	±	Strongly positive, 3+
	5	10	+	0	0	+	+	±	Strongly positive, 5+
	4	10	+	0	0	+	+	±	Strongly positive, 5+
43	5	5	+	+	tr	+	+	±	Weakly positive, 1+
	6	5	+	+	tr	+	+	±	Weakly positive, 1+
	5	10	+	+	tr	+	+	±	Weakly positive, 1+
	6	10	+	+	tr	+	+	±	Weakly positive, 1+
44	5	5	+	+	0	+	+	±	Moderately positive, 2+
	6	5	+	+	0	+	+	±	Moderately positive, 2+
	5	10	+	+	0	+	+	±	Moderately positive, 2+
	6	10	+	+	0	+	+	±	Moderately positive, 2+
45	5	5	+	+	0	+	+	±	Moderately positive, 2+
	6	5	+	+	0	+	+	±	Moderately positive, 2+
	5	10	+	+	0	+	+	±	Moderately positive, 2+
	6	10	+	+	0	+	+	±	Moderately positive, 2+
46	5	5	+	tr	0	+	+	±	Strongly positive, 4+
	6	5	+	±	0	+	+	±	Strongly positive, 3+
	5	10	+	tr	0	+	+	±	Strongly positive, 4+
	6	10	+	tr	0	+	+	±	Strongly positive, 4+
47	5	5	+	+	0	+	+	±	Moderately positive, 2+
	6	5	+	+	0	+	+	±	Moderately positive, 2+
	5	10	+	+	0	+	+	±	Moderately positive, 2+
	6	10	+	+	0	+	+	±	Moderately positive, 2+
48	5	5	+	±	0	+	+	±	Strongly positive, 3+
	6	5	+	±	0	+	+	±	Strongly positive, 3+
	5	10	+	tr	0	+	+	±	Strongly positive, 4+
	6	10	+	tr	0	+	+	±	Strongly positive, 4+
49	5	5	+	±	0	+	+	±	Strongly positive, 3+
	7	5	+	±	0	+	+	±	Strongly positive, 3+
	5	10	+	tr	0	+	+	±	Strongly positive, 4+
	7	10	+	±	0	+	+	±	Strongly positive, 3+
50	5	5	+	tr	0	+	+	±	Strongly positive, 4+
	7	5	+	±	0	+	+	±	Strongly positive, 3+
	5	10	±	0	0	+	+	±	Strongly positive, 6+
	7	10	+	tr	0	+	+	±	Strongly positive, 4+
51	5	5	+	+	0	+	+	±	Moderately positive, 2+
	7	5	+	+	0	+	+	±	Moderately positive, 2+
	5	10	+	±	0	+	+	±	Strongly positive, 3+
	7	10	+	+	0	+	+	±	Moderately positive, 2+
52	5	5	+	0	0	+	+	±	Strongly positive, 5+
	7	5	+	tr	0	+	+	±	Strongly positive, 4+
	5	10	+	0	0	+	+	±	Strongly positive, 5+
	7	10	+	tr	0	+	+	±	Strongly positive, 4+
53	5	5	+	tr	0	+	+	±	Strongly positive, 4+
	7	5	+	tr	0	+	+	±	Strongly positive, 4+
	5	10	±	0	0	+	+	±	Strongly positive, 6+
	7	10	+	tr	0	+	+	±	Strongly positive, 4+

TABLE 2—Continued

INCUBATION AT 1 C., 2 C., 3 C., 4 C., 6 C., 7 C., 8 C. AND 9 C. COMPARED WITH
INCUBATION AT 5 C.

Number of Serum	Incubation Tempera- ture, C.	Incubation Time, Hours	Readings						Results	
			Antigen Tubes			Control Tubes				
			1	2	3	1'	2'	3'		
54	5	5	+	0	0	+	+	±	Strongly positive,	5+
	7	5	+	tr	0	+	+	±	Strongly positive,	4+
	5	10	tr	0	0	+	+	±	Strongly positive,	8+
	7	10	+	0	0	+	+	±	Strongly positive,	5+
55	5	5	+	±	0	+	+	±	Strongly positive,	3+
	8	5	+	+	0	+	+	±	Moderately positive,	2+
	5	10	+	tr	0	+	+	±	Strongly positive,	4+
	8	10	+	±	0	+	+	±	Strongly positive,	3+
56	5	5	+	±	0	+	+	±	Strongly positive,	3+
	8	5	+	+	tr	+	+	±	Weakly positive,	1+
	5	10	+	tr	0	+	+	±	Strongly positive,	4+
	8	10	+	+	0	+	+	±	Moderately positive,	2+
57	5	5	+	±	0	+	+	±	Strongly positive,	3+
	8	5	+	+	0	+	+	±	Moderately positive,	2+
	5	10	+	tr	0	+	+	±	Strongly positive,	4+
	8	10	+	±	0	+	+	±	Strongly positive,	3+
58	5	5	+	±	0	+	+	±	Strongly positive,	3+
	8	5	+	+	tr	+	+	±	Weakly positive,	1+
	5	10	+	+	0	+	+	±	Strongly positive,	3+
	8	10	+	+	0	+	+	±	Moderately positive,	2+
59	5	5	+	+	0	+	+	±	Moderately positive,	2+
	8	5	+	+	0	+	+	±	Moderately positive,	2+
	5	10	+	±	0	+	+	±	Strongly positive,	3+
	8	10	+	+	0	+	+	±	Moderately positive,	2+
60	5	5	+	+	0	+	+	±	Moderately positive,	2+
	8	5	+	+	tr	+	+	±	Weakly positive,	1+
	5	10	+	+	0	+	+	±	Moderately positive,	2+
	8	10	+	+	tr	+	+	±	Weakly positive,	1+
61	5	5	+	tr	0	+	+	±	Strongly positive,	4+
	9	5	+	±	0	+	+	±	Strongly positive,	3+
	5	10	+	0	0	+	+	±	Strongly positive,	5+
	9	10	+	tr	0	+	+	±	Strongly positive,	4+
62	5	5	+	±	0	+	+	±	Strongly positive,	3+
	9	5	+	+	0	+	+	±	Moderately positive,	2+
	5	10	+	0	0	+	+	±	Strongly positive,	5+
	9	10	+	±	0	+	+	±	Strongly positive,	3+
63	5	5	+	0	0	+	+	±	Strongly positive,	5+
	9	5	+	tr	0	+	+	±	Strongly positive,	4+
	5	10	tr	0	0	+	+	±	Strongly positive,	8+
	9	10	+	tr	0	+	+	±	Strongly positive,	4+
64	5	5	+	tr	0	+	+	±	Strongly positive,	4+
	9	5	+	±	0	+	+	±	Strongly positive,	3+
	5	10	+	0	0	+	+	±	Strongly positive,	5+
	9	10	+	±	0	+	+	±	Strongly positive,	3+
65	5	5	+	±	0	+	+	±	Strongly positive,	3+
	9	5	+	±	0	+	+	±	Strongly positive,	3+
	5	10	+	0	0	+	+	±	Strongly positive,	5+
	9	10	+	±	0	+	+	±	Strongly positive,	3+
66	5	5	+	±	0	+	+	±	Strongly positive,	3+
	9	5	+	+	0	+	+	±	Moderately positive,	2+
	5	10	+	tr	0	+	+	±	Strongly positive,	4+
	9	10	+	+	0	+	+	±	Moderately positive,	2+

The results obtained by comparing incubation at 1 C., 2 C., 3 C., 4 C., 6 C., 7 C., 8 C. and 9 C. for 5 hours and 10 hours with incubation at 5 C. for 5 and 10 hours are shown in Table 2. Incubation at 1 C., 2 C., 3 C. and 4 C. gave stronger positive results than did incubation at 5 C. and incubation at 6 C., 7 C., 8 C. and 9 C. gave weaker positive results than did incubation at 5 C. Incubation for 10 hours gave stronger positive results than did incubation for 5 hours.

TEST 3

Serums 67 to 84, inclusive, incubation at 3 C., 1 C. and 0.5 C. for 5 hours and for 10 hours was compared with incubation at 2 C. for 5 and 10 hours.

TABLE 3
INCUBATION AT 3 C., 1 C. AND 0.5 C. COMPARED WITH INCUBATION AT 2 C.

Number of Serum	Incubation Tempera- ture, C.	Incubation Time, Hours	Readings						Results	
			Antigen Tubes			Control Tubes				
			1	2	3	1'	2'	3'		
67	2	5	+	0	0	+	+	±	Strongly positive,	5+
	3	5	+	tr	0	+	+	±	Strongly positive,	4+
	2	10	±	0	0	+	+	±	Strongly positive,	6+
	3	10	+	0	0	+	+	±	Strongly positive,	5+
68	2	5	+	tr	0	+	+	±	Strongly positive,	4+
	3	5	+	±	0	+	+	±	Strongly positive,	3+
	2	10	+	0	0	+	+	±	Strongly positive,	5+
	3	10	+	tr	0	+	+	±	Strongly positive,	4+
69	2	5	+	0	0	+	+	±	Strongly positive,	5+
	3	5	+	tr	0	+	+	±	Strongly positive,	4+
	2	10	±	0	0	+	+	±	Strongly positive,	6+
	3	10	+	0	0	+	+	±	Strongly positive,	5+
70	2	5	+	0	0	+	+	±	Strongly positive,	5+
	3	5	+	tr	0	+	+	±	Strongly positive,	4+
	2	10	±	0	0	+	+	±	Strongly positive,	6+
	3	10	+	tr	0	+	+	±	Strongly positive,	4+
71	2	5	+	+	0	+	+	±	Moderately positive,	2+
	3	5	+	+	0	+	+	±	Moderately positive,	2+
	2	10	+	±	0	+	+	±	Strongly positive,	3+
	3	10	+	±	0	+	+	±	Strongly positive,	3+
72	2	5	+	+	0	+	+	±	Moderately positive,	2+
	3	5	+	+	0	+	+	±	Moderately positive,	2+
	2	10	+	±	0	+	+	±	Strongly positive,	3+
	3	10	+	±	0	+	+	±	Strongly positive,	3+
73	2	5	+	+	tr	+	+	±	Weakly positive,	1+
	1	5	+	+	tr	+	+	±	Weakly positive,	1+
	2	10	+	+	0	+	+	±	Moderately positive,	2+
	1	10	+	+	0	+	+	±	Moderately positive,	2+
74	2	5	+	+	tr	+	+	±	Weakly positive,	1+
	1	5	+	+	tr	+	+	±	Weakly positive,	1+
	2	10	+	+	tr	+	+	±	Weakly positive,	1+
	1	10	+	+	0	+	+	±	Moderately positive,	2+

TABLE 3—Continued

INCUBATION AT 3 C., 1 C. AND 0.5 C. COMPARED WITH INCUBATION AT 2 C.

Number of Serum	Incubation Tempera- ture, C.	Incubation Time, Hours	Readings						Results
			Antigen Tubes			Control Tubes			
			1	2	3	1'	2'	3'	
75	2	5	+	+	tr	+	+	±	Weakly positive, 1+
	1	5	+	+	tr	+	+	±	Weakly positive, 1+
	2	10	+	+	tr	+	+	±	Weakly positive, 1+
	1	10	+	+	0	+	+	±	Moderately positive, 2+
76	2	5	+	±	0	+	+	±	Strongly positive, 3+
	1	5	+	tr	0	+	+	±	Strongly positive, 4+
	2	10	+	tr	0	+	+	±	Strongly positive, 4+
	1	10	+	0	0	+	+	±	Strongly positive, 5+
77	2	5	+	+	0	+	+	±	Moderately positive, 2+
	1	5	+	+	0	+	+	±	Moderately positive, 2+
	2	10	+	+	0	+	+	±	Moderately positive, 2+
	1	10	+	±	0	+	+	±	Strongly positive, 3+
78	2	5	+	±	0	+	+	±	Strongly positive, 3+
	1	5	+	tr	0	+	+	±	Strongly positive, 4+
	2	10	+	0	0	+	+	±	Strongly positive, 5+
	1	10	+	0	0	+	+	±	Strongly positive, 5+
79	2	5	+	tr	0	+	+	±	Strongly positive, 4+
	0.5	5	+	tr	0	+	+	±	Strongly positive, 4+
	2	10	+	tr	0	+	+	±	Strongly positive, 4+
	0.5	10	+	0	0	+	+	±	Strongly positive, 5+
80	2	5	+	±	0	+	+	±	Strongly positive, 3+
	0.5	5	+	±	0	+	+	±	Strongly positive, 3+
	2	10	+	±	0	+	+	±	Strongly positive, 3+
	0.5	10	+	tr	0	+	+	±	Strongly positive, 4+
81	2	5	+	tr	0	+	+	±	Strongly positive, 4+
	0.5	5	+	0	0	+	+	±	Strongly positive, 5+
	2	10	+	0	0	+	+	±	Strongly positive, 5+
	0.5	10	±	0	0	+	+	±	Strongly positive, 6+
82	2	5	+	+	0	+	+	±	Moderately positive, 2+
	0.5	5	+	+	0	+	+	±	Moderately positive, 2+
	2	10	+	±	0	+	+	±	Strongly positive, 3+
	0.5	10	+	tr	0	+	+	±	Strongly positive, 4+
83	2	5	+	+	0	+	+	±	Moderately positive, 2+
	0.5	5	+	+	0	+	+	±	Moderately positive, 2+
	2	10	+	+	0	+	+	±	Moderately positive, 2+
	0.5	10	+	±	0	+	+	±	Strongly positive, 3+
84	2	5	+	+	tr	+	+	±	Weakly positive, 1+
	0.5	5	+	+	tr	+	+	±	Weakly positive, 1+
	2	10	+	+	tr	+	+	±	Weakly positive, 1+
	0.5	10	+	+	0	+	+	±	Moderately positive, 2+

Table 3 shows the results obtained by comparing incubation at 3 C., 1 C. and 0.5 C. for 5 hours and 10 hours with incubation at 2 C. for 5 and 10 hours. Incubation at 3 C. gave weaker positive results than did incubation at 2 C. and incubation at 1 C. and at 0.5 C. gave stronger positive results than did incubation at 2 C. Incubation for 10 hours gave stronger positive results than did incubation for 5 hours.

TEST 4

Incubation at 0.5 C. for 5 hours and for 10 hours compared with incubation at 1 C. for 5 and 10 hours on 12 serums—85 to 96, inclusive.

TABLE 4
INCUBATION AT 0.5 C. COMPARED WITH INCUBATION AT 1 C.

Number of Serum	Incubation Tempera- ture, C.	Incubation Time, Hours	Readings						Results	
			Antigen Tubes			Control Tubes				
			1	2	3	1'	2'	3'		
85	1	5	tr	0	0	+	+	±	Strongly positive,	8+
	0.5	5	tr	0	0	+	+	±	Strongly positive,	8+
	1	10	0	0	0	+	+	±	Strongly positive,	10+
	0.5	10	0	0	0	+	+	±	Strongly positive,	10+
86	1	5	±	0	0	+	+	±	Strongly positive,	6+
	0.5	5	±	0	0	+	+	±	Strongly positive,	6+
	1	10	tr	0	0	+	+	±	Strongly positive,	8+
	0.5	10	tr	0	0	+	+	±	Strongly positive,	8+
87	1	5	tr	0	0	+	+	±	Strongly positive,	8+
	0.5	5	tr	0	0	+	+	±	Strongly positive,	8+
	1	10	0	0	0	+	+	±	Strongly positive,	10+
	0.5	10	0	0	0	+	+	±	Strongly positive,	10+
88	1	5	±	0	0	+	+	±	Strongly positive,	6+
	0.5	5	±	0	0	+	+	±	Strongly positive,	6+
	1	10	tr	0	0	+	+	±	Strongly positive,	8+
	0.5	10	tr	0	0	+	+	±	Strongly positive,	8+
89	1	5	+	+	0	+	+	±	Moderately positive,	2+
	0.5	5	+	+	0	+	+	±	Moderately positive,	2+
	1	10	+	±	0	+	+	±	Strongly positive,	3+
	0.5	10	+	±	0	+	+	±	Strongly positive,	3+
90	1	5	+	+	0	+	+	±	Moderately positive,	2+
	0.5	5	+	+	0	+	+	±	Moderately positive,	2+
	1	10	+	±	0	+	+	±	Strongly positive,	3+
	0.5	10	+	±	0	+	+	±	Strongly positive,	3+
91	1	5	+	+	0	+	+	±	Moderately positive,	2+
	0.5	5	+	+	0	+	+	±	Moderately positive,	2+
	1	10	+	±	0	+	+	±	Strongly positive,	3+
	0.5	10	+	±	0	+	+	±	Strongly positive,	3+
92	1	5	+	+	0	+	+	±	Moderately positive,	2+
	0.5	5	+	+	0	+	+	±	Moderately positive,	2+
	1	10	+	±	0	+	+	±	Strongly positive,	3+
	0.5	10	+	±	0	+	+	±	Strongly positive,	3+
93	1	5	+	+	0	+	+	±	Moderately positive,	2+
	0.5	5	+	+	0	+	+	±	Moderately positive,	2+
	1	10	+	tr	0	+	+	±	Strongly positive,	4+
	0.5	10	+	tr	0	+	+	±	Strongly positive,	4+
94	1	5	+	+	tr	+	+	±	Weakly positive,	1+
	0.5	5	+	+	tr	+	+	±	Weakly positive,	1+
	1	10	+	±	0	+	+	±	Strongly positive,	3+
	0.5	10	+	±	0	+	+	±	Strongly positive,	3+
95	1	5	+	+	tr	+	+	±	Weakly positive,	1+
	0.5	5	+	+	tr	+	+	±	Weakly positive,	1+
	1	10	+	+	0	+	+	±	Moderately positive,	2+
	0.5	10	+	+	0	+	+	±	Moderately positive,	2+
96	1	5	+	+	tr	+	+	±	Weakly positive,	1+
	0.5	5	+	+	tr	+	+	±	Weakly positive,	1+
	1	10	+	±	0	+	+	±	Strongly positive,	3+
	0.5	10	+	±	0	+	+	±	Strongly positive,	3+

Table 4 shows the results obtained by comparing incubation at 0.5 C. for 5 and 10 hours with incubation at 1 C. for 5 and 10 hours—serums 85 to 96, inclusive. The results obtained with incubation at 0.5 C. were identical with those obtained with incubation at 1 C. Ten-hour incubation at both of these temperatures gave stronger positive results than did 5-hour incubation.

TEST 5

Incubation for 15 hours at 1 C. compared with incubation for 24 hours at 1 C.—serums 97 to 120, inclusive.

TABLE 5

FIFTEEN-HOUR INCUBATION AT 1 C. COMPARED WITH TWENTY-FOUR-HOUR INCUBATION AT 1 C.

Number of Serum	Incubation Tempera- ture, C.	Incubation Time, Hours	Readings						Results	
			Antigen Tubes			Control Tubes				
			1	2	3	1'	2'	3'		
97	1	15	±	0	0	+	+	±	Strongly positive, Strongly positive,	6+ 6+
	1	24	tr	0	0	+	+	tr		
98	1	15	tr	0	0	+	+	±	Strongly positive, Strongly positive,	8+ 8+
	1	24	0	0	0	+	+	±		
99	1	15	tr	0	0	+	+	±	Strongly positive, Strongly positive,	8+ 10+
	1	24	0	0	0	+	+	tr		
100	1	15	tr	0	0	+	+	±	Strongly positive, Strongly positive,	8+ 10+
	1	24	0	0	0	+	+	±		
101	1	15	±	0	0	+	+	±	Strongly positive, Strongly positive,	6+ 6+
	1	24	tr	0	0	+	+	tr		
102	1	15	tr	0	0	+	+	±	Strongly positive, Strongly positive,	8+ 8+
	1	24	0	0	0	+	+	tr		
103	1	15	tr	0	0	+	+	±	Strongly positive, Strongly positive,	8+ 10+
	1	24	0	0	0	+	+	±		
104	1	15	±	0	0	+	+	±	Strongly positive, Strongly positive,	6+ 8+
	1	24	tr	0	0	+	+	±		
105	1	15	tr	0	0	+	+	±	Strongly positive, Strongly positive,	8+ 10+
	1	24	0	0	0	+	+	±		
106	1	15	±	0	0	+	+	±	Strongly positive, Strongly positive,	6+ 8+
	1	24	0	0	0	+	+	tr		
107	1	15	+	tr	0	+	+	±	Strongly positive, Strongly positive,	4+ 5+
	1	24	±	0	0	+	+	tr		
108	1	15	±	0	0	+	+	±	Strongly positive, Strongly positive,	6+ 8+
	1	24	0	0	0	+	+	tr		
109	1	15	tr	tr	0	+	+	±	Unfit Unfit	
	1	24	0	0	0	tr	±	tr		
110	1	15	±	tr	0	+	+	±	Unfit Strongly positive,	
	1	24	tr	0	0	+	+	tr		
111	1	15	0	0	0	+	+	±	Strongly positive, Strongly positive,	10+ 10+
	1	24	0	0	0	+	+	±		
112	1	15	+	±	0	+	+	±	Strongly positive, Unfit	3+
	1	24	tr	tr	0	+	+	±		

TABLE 5—Continued

FIFTEEN-HOUR INCUBATION AT 1 C. COMPARED WITH TWENTY-FOUR-HOUR INCUBATION AT 1 C.

Number of Serum	Incubation Temperature, C.	Incubation Time, Hours	Readings						Results	
			Antigen Tubes			Control Tubes				
			1	2	3	1'	2'	3'		
113	1	15	+	\pm	0	+	+	\pm	Strongly positive, 3+	Strongly positive, 3+
	1	24	+	tr	0	+	+	tr		
114	1	15	+	\pm	0	+	+	\pm	Strongly positive, 3+	Strongly positive, 8+
	1	24	0	0	0	+	+	tr		
115	1	15	+	\pm	tr	+	+	\pm	Unfit?	Unfit
	1	24	+	tr	tr	+	+	\pm		
116	1	15	tr	0	0	+	+	\pm	Strongly positive, 8+	Unfit
	1	24	0	0	0	+	\pm	\pm		
117	1	15	0	0	0	tr	\pm	tr	Unfit	Unfit
	1	24	0	0	0	tr	\pm	\pm		
118	1	15	0	0	0	+	+	\pm	Strongly positive, 10+	Strongly positive, 8+
	1	24	0	0	0	+	+	tr		
119	1	15	0	0	0	tr	\pm	\pm	Unfit	Unfit
	1	24	0	0	0	0	\pm	tr		
120	1	15	0	0	0	+	+	tr	Strongly positive, 8+	Unfit
	1	24	0	0	0	tr	\pm	tr		

The results obtained by comparing incubation for 15 hours at 1 C. with incubation for 24 hours at 1 C. are shown in Table 5. With serums 97 to 108, inclusive, the results were good, and with incubation for 24 hours the results were frequently stronger positive than with incubation for 15 hours, showing that complement binding was not always completed in 15 hours. With serums 109 to 120, inclusive, the results were poor. As these 12 serums were tested with the same lot of complement, and as such poor results were not obtained otherwise with incubation for 15 hours the complement is blamed for these poor results.

TEST 6

Fifteen-hour incubation at 1 C. was compared with 10-hour incubation at 1 C. on 24 normal human serums (121 to 144, inclusive).

TABLE 6

FIFTEEN-HOUR INCUBATION AT 1 C. COMPARED WITH TEN-HOUR INCUBATION AT 1 C. NORMAL SERUM

Number of Serum	Incubation Tempera- ture, C.	Incubation Time, Hours	Readings						Results
			Antigen Tubes			Control Tubes			
			1	2	3	1'	2'	3'	
121	1	10	+	+	±	+	+	±	Negative, —
	1	15	+	+	±	+	+	±	Negative, —
122	1	10	+	+	±	+	+	±	Negative, —
	1	15	+	+	±	+	+	±	Negative, —

TABLE 6—Continued

FIFTEEN-HOUR INCUBATION AT 1 C. COMPARED WITH TEN-HOUR INCUBATION AT 1 C.
NORMAL SERUM

Number of Serum	Incubation Tempera- ture, C.	Incubation Time, Hours	Readings						Results
			Antigen Tubes			Control Tubes			
			1	2	3	1'	2'	3'	
123	1	10	+	+	tr	+	+	tr	Negative, —
	1	15	+	+	tr	+	+	tr	Negative, —
124	1	10	+	+	±	+	+	±	Negative, —
	1	15	+	+	tr	+	+	tr	Negative, —
125	1	10	+	+	±	+	+	±	Negative, —
	1	15	+	+	±	+	+	±	Negative, —
126	1	10	+	+	±	+	+	±	Negative, —
	1	15	+	+	tr	+	+	tr	Negative, —
127	1	10	+	+	tr	+	+	tr	Negative, —
	1	15	+	+	tr	+	+	tr	Negative, —
128	1	10	+	+	±	+	+	±	Negative, —
	1	15	+	+	±	+	+	±	Negative, —
129	1	10	+	+	±	+	+	±	Negative, —
	1	15	+	+	±	+	+	±	Negative, —
130	1	10	+	+	±	+	+	±	Negative, —
	1	15	+	+	tr	+	+	tr	Negative, —
131	1	10	+	+	tr	+	+	tr	Negative, —
	1	15	+	+	tr	+	+	tr	Negative, —
132	1	10	+	+	±	+	+	±	Negative, —
	1	15	+	+	tr	+	+	tr	Negative, —
133	1	10	+	+	±	+	+	±	Negative, —
	1	15	+	+	±	+	+	±	Negative, —
134	1	10	+	+	±	+	+	±	Negative, —
	1	15	+	+	±	+	+	±	Negative, —
135	1	10	+	+	±	+	+	±	Negative, —
	1	15	+	+	±	+	+	±	Negative, —
136	1	10	+	+	±	+	+	±	Negative, —
	1	15	+	+	±	+	+	±	Negative, —
137	1	10	+	+	±	+	+	±	Negative, —
	1	15	+	+	±	+	+	±	Negative, —
138	1	10	+	+	±	+	+	±	Negative, —
	1	15	+	+	±	+	+	±	Negative, —
139	1	10	+	+	±	+	+	±	Negative, —
	1	15	+	+	±	+	+	±	Negative, —
140	1	10	+	+	tr	+	+	tr	Negative, —
	1	15	+	+	tr	+	+	tr	Negative, —
141	1	10	+	+	±	+	+	±	Negative, —
	1	15	+	+	±	+	+	±	Negative, —
142	1	10	+	+	tr	+	+	tr	Negative, —
	1	15	+	+	0	+	+	0	Negative, —
143	1	10	+	+	tr	+	+	tr	Negative, —
	1	15	+	+	tr	+	+	tr	Negative, —
144	1	10	+	+	tr	+	+	tr	Negative, —
	1	15	+	+	tr	+	+	tr	Negative, —

Table 6 shows the results obtained by comparing 15-hour incubation at 1 C. with 10-hour incubation at 1 C. on 24 specimens of normal human serum. All of the results were negative.

TEST 7

On Serums 145 to 160, inclusive, acetone insoluble antigen of beef heart (A. I. B. H.) was compared with alcoholic extract of beef heart (A. E. B. H.). Incubation at 1 C. and at 37 C. for 1 hour and for 10 hours.

TABLE 7

ACETONE INSOLUBLE ANTIGEN COMPARED WITH CRUDE ALCOHOLIC EXTRACT, INCUBATION AT 1 C. AND 37 C. FOR 1 HOUR AND 10 HOURS

Number of Serum	Kind of Antigen	Incuba- tion Temper- ature, C.	Incuba- tion Time, Hours	Readings						Results	
				Antigen Tubes			Control Tubes				
				1	2	3	1'	2'	3'		
145	A.E.B.H.	1	1	+	+	±	+	+	±	Negative,	—
	A.I.B.H.	1	1	+	+	±	+	+	±	Negative,	—
	A.E.B.H.	37	1	+	+	±	+	+	±	Negative,	—
	A.I.B.H.	37	1	+	+	±	+	+	±	Negative,	—
	A.E.B.H.	1	10	+	+	±	+	+	±	Negative,	—
	A.I.B.H.	1	10	+	+	±	+	+	±	Negative,	—
	A.E.B.H.	37	10	+	+	tr	+	+	tr	Negative,	—
	A.I.B.H.	37	10	+	+	tr	+	+	tr	Negative,	—
146	A.E.B.H.	1	1	+	+	±	+	+	±	Negative,	—
	A.I.B.H.	1	1	+	+	±	+	+	±	Negative,	—
	A.E.B.H.	37	1	+	+	±	+	+	±	Negative,	—
	A.I.B.H.	37	1	+	+	±	+	+	±	Negative,	—
	A.E.B.H.	1	10	+	±	0	+	+	±	Strongly positive,	3+
	A.I.B.H.	1	10	+	+	±	+	+	±	Negative,	—
	A.E.B.H.	37	10	+	+	tr	+	+	tr	Negative,	—
	A.I.B.H.	37	10	+	+	tr	+	+	tr	Negative,	—
147	A.E.B.H.	1	1	+	+	±	+	+	±	Negative,	—
	A.I.B.H.	1	1	+	+	±	+	+	±	Negative,	—
	A.E.B.H.	37	1	+	+	±	+	+	±	Negative,	—
	A.I.B.H.	37	1	+	+	±	+	+	±	Negative,	—
	A.E.B.H.	1	10	+	tr	0	+	+	±	Strongly positive,	4+
	A.I.B.H.	1	10	+	+	±	+	+	±	Negative,	—
	A.E.B.H.	37	10	+	+	tr	+	+	tr	Negative,	—
	A.I.B.H.	37	10	+	+	tr	+	+	tr	Negative,	—
148	A.E.B.H.	1	1	+	+	tr	+	+	±	Weakly positive,	1+
	A.I.B.H.	1	1	+	+	±	+	+	±	Negative,	—
	A.E.B.H.	37	1	+	+	tr	+	+	±	Weakly positive,	1+
	A.I.B.H.	37	1	+	+	±	+	+	±	Negative,	—
	A.E.B.H.	1	10	+	tr	0	+	+	±	Strongly positive,	4+
	A.I.B.H.	1	10	+	+	±	+	+	±	Negative,	—
	A.E.B.H.	37	10	+	+	0	+	+	tr	Weakly positive,	1+
	A.I.B.H.	37	10	+	+	tr?	+	+	tr	Faintly positive,	±
149	A.E.B.H.	1	1	+	+	tr	+	+	±	Weakly positive,	1+
	A.I.B.H.	1	1	+	+	±	+	+	±	Negative,	—
	A.E.B.H.	37	1	+	+	±?	+	+	±	Faintly positive,	±
	A.I.B.H.	37	1	+	+	±	+	+	±	Negative,	—
	A.E.B.H.	1	10	+	+	0	+	+	±	Moderately positive,	2+
	A.I.B.H.	1	10	+	+	±	+	+	±	Negative,	—
	A.E.B.H.	37	10	+	+	tr?	+	+	tr	Faintly positive,	±
	A.I.B.H.	37	10	+	+	tr	+	+	tr	Negative,	—

TABLE 7—Continued

ACETONE INSOLUBLE ANTIGEN COMPARED WITH CRUDE ALCOHOLIC EXTRACT, INCUBATION AT 1 C. AND 37 C. FOR 1 HOUR AND 10 HOURS

Number of Serum	Kind of Antigen	Incubation Temperature, C.	Incubation Time, Hours	Readings						Results	
				Antigen Tubes			Control Tubes				
				1	2	3	1'	2'	3'		
150	A.E.B.H.	1	1	+	+	±	+	+	±	Negative,	—
	A.I.B.H.	1	1	+	+	±	+	+	±	Negative,	—
	A.E.B.H.	37	1	+	+	±	+	+	±	Negative,	—
	A.I.B.H.	37	1	+	+	±	+	+	±	Negative,	—
	A.E.B.H.	1	10	+	±	0	+	+	±	Strongly positive,	3+
	A.I.B.H.	1	10	+	+	±	+	+	±	Negative,	—
	A.E.B.H.	37	10	+	+	tr?	+	+	tr	Faintly positive,	±
151	A.I.B.H.	37	10	+	+	tr	+	+	tr	Negative,	—
	A.E.B.H.	1	1	+	+	±	+	+	±	Negative,	—
	A.I.B.H.	1	1	+	+	±	+	+	±	Negative,	—
	A.E.B.H.	37	1	+	+	±	+	+	±	Negative,	—
	A.I.B.H.	37	1	+	+	±	+	+	±	Negative,	—
	A.E.B.H.	1	10	+	+	tr	+	+	±	Weakly positive,	1+
	A.I.B.H.	1	10	+	+	±	+	+	±	Negative,	—
152	A.E.B.H.	37	10	+	+	tr	+	+	tr	Negative,	—
	A.I.B.H.	37	10	+	+	tr	+	+	tr	Negative,	—
	A.E.B.H.	1	1	+	+	±	+	+	±	Negative,	—
	A.I.B.H.	1	1	+	+	±	+	+	±	Negative,	—
	A.E.B.H.	37	1	+	+	±	+	+	±	Negative,	—
	A.I.B.H.	37	1	+	+	±	+	+	±	Negative,	—
	A.E.B.H.	1	10	+	+	tr	+	+	±	Weakly positive,	1+
153	A.I.B.H.	1	10	+	+	±	+	+	±	Negative,	—
	A.E.B.H.	37	10	+	+	tr	+	+	tr	Negative,	—
	A.I.B.H.	37	10	+	+	tr	+	+	tr	Negative,	—
	A.E.B.H.	1	1	+	+	±	+	+	±	Negative,	—
	A.I.B.H.	1	1	+	+	±	+	+	±	Negative,	—
	A.E.B.H.	37	1	+	+	±	+	+	±	Negative,	—
	A.I.B.H.	37	1	+	+	±	+	+	±	Negative,	—
154	A.E.B.H.	1	10	+	+	tr	+	+	±	Weakly positive,	1+
	A.I.B.H.	1	10	+	+	±	+	+	±	Negative,	—
	A.E.B.H.	37	10	+	+	tr	+	+	tr	Negative,	—
	A.I.B.H.	37	10	+	+	tr	+	+	tr	Negative,	—
	A.E.B.H.	1	1	+	+	±	+	+	±	Negative,	—
	A.I.B.H.	1	1	+	+	±	+	+	±	Negative,	—
	A.E.B.H.	37	1	+	+	±	+	+	±	Negative,	—
155	A.I.B.H.	37	1	+	+	±	+	+	±	Negative,	—
	A.E.B.H.	1	10	+	+	tr	+	+	±	Weakly positive,	1+
	A.I.B.H.	1	10	+	+	±	+	+	±	Negative,	—
	A.E.B.H.	37	10	+	+	tr	+	+	tr	Negative,	—
	A.I.B.H.	37	10	+	+	tr	+	+	tr	Negative,	—
	A.E.B.H.	1	1	+	±	0	+	+	±	Strongly positive,	3+
	A.I.B.H.	1	1	+	+	±	+	+	±	Negative,	—
156	A.E.B.H.	37	1	+	+	tr	+	+	±	Weakly positive,	1+
	A.I.B.H.	37	1	+	+	±	+	+	±	Negative,	—
	A.E.B.H.	1	10	±	0	0	+	+	±	Strongly positive,	6+
	A.I.B.H.	1	10	+	+	±	+	+	±	Negative,	—
	A.E.B.H.	37	10	+	±	0	+	+	tr	Moderately positive,	2+
	A.I.B.H.	37	10	+	+	0	+	+	tr	Weakly positive,	1+
	A.E.B.H.	1	1	+	±	0	+	+	±	Strongly positive,	10+
157	A.I.B.H.	1	1	+	+	±	+	+	±	Negative,	—
	A.E.B.H.	37	1	+	+	±	+	+	±	Negative,	—
	A.I.B.H.	37	1	+	+	±	+	+	±	Negative,	—
	A.E.B.H.	1	10	0	0	0	+	+	±	Strongly positive,	10+
	A.I.B.H.	1	10	+	+	±	+	+	±	Negative,	—
	A.E.B.H.	37	10	+	±	0	+	+	tr	Moderately positive,	2+
	A.I.B.H.	37	10	+	+	0	+	+	tr	Weakly positive,	1+

TABLE 7—Continued

ACETONE INSOLUBLE ANTIGEN COMPARED WITH CRUDE ALCOHOLIC EXTRACT, INCUBATION AT
1 C. AND 37 C. FOR 1 HOUR AND 10 HOURS

Number of Serum	Kind of Antigen	Incuba- tion Temper- ature, C.	Incuba- tion Time, Hours	Readings						Results
				Antigen Tubes			Control Tubes			
				1	2	3	1'	2'	3'	
157 Dilution 1:5	A.E.B.H.	1	1	0	0	0	+	+	±	10+ × 5 = 50+
	A.I.B.H.	1	1	+	±	0	+	+	±	3+ × 5 = 15+
	A.E.B.H.	37	1	+	±	0	+	+	±	3+ × 5 = 15+
	A.I.B.H.	37	1	+	+	tr	+	+	±	1+ × 5 = 5+
	A.E.B.H.	1	10	0	0	0	+	+	±	10+ × 5 = 50+
	A.I.B.H.	1	10	tr	0	0	+	+	±	8+ × 5 = 40+
	A.E.B.H.	37	10	+	±	0	+	+	±	3+ × 5 = 15+
	A.I.B.H.	37	10	+	+	0	+	+	±	2+ × 5 = 10+
Dilution 1:25	A.E.B.H.	1	1	+	±	0	+	+	±	3+ × 25 = 75+
	A.E.B.H.	1	1	+	+	±	+	+	±	Negative, —
	A.E.B.H.	37	1	+	+	±	+	+	±	Negative, —
	A.I.B.H.	37	1	+	+	±	+	+	±	Negative, —
	A.E.B.H.	1	10	tr	0	0	+	+	±	8+ × 25 = 200+
	A.I.B.H.	1	10	+	+	±	+	+	±	Negative, —
	A.E.B.H.	37	10	+	+	tr	+	+	±	1+ × 25 = 25+
	A.I.B.H.	37	10	+	+	tr	+	+	±	1+ × 25 = 25+
157 (cont.) Dilution 1:125	A.E.B.H.	1	1	+	+	±	+	+	±	Negative, —
	A.I.B.H.	1	1	+	+	±	+	+	±	Negative, —
	A.E.B.H.	37	1	+	+	±	+	+	±	Negative, —
	A.I.B.H.	37	1	+	+	±	+	+	±	Negative, —
	A.E.B.H.	1	10	+	+	±	+	+	±	Negative, —
	A.I.B.H.	1	10	+	+	±	+	+	±	Negative, —
	A.E.B.H.	37	10	+	+	±	+	+	±	Negative, —
	A.I.B.H.	37	10	+	+	±	+	+	±	Negative, —
158 Dilution 1:5	A.E.B.H.	1	1	tr	0	0	+	+	±	8+ × 5 = 40+
	A.I.B.H.	1	1	+	tr	0	+	+	±	4+ × 5 = 20+
	A.E.B.H.	37	1	0	0	0	+	+	tr	8+ × 5 = 40+
	A.I.B.H.	37	1	0	0	0	+	+	tr	8+ × 5 = 40+
	A.E.B.H.	1	10	0	0	0	+	+	±	10+ × 5 = 50+
	A.I.B.H.	1	10	tr	0	0	+	+	±	8+ × 5 = 40+
	A.E.B.H.	37	10	0	0	0	+	+	tr	8+ × 5 = 40+
	A.I.B.H.	37	10	0	0	0	+	+	tr	8+ × 5 = 40+
Dilution 1:25	A.E.B.H.	1	1	+	+	0	+	+	±	2+ × 25 = 50+
	A.I.B.H.	1	1	+	+	tr	+	+	±	1+ × 25 = 25+
	A.E.B.H.	37	1	+	0	0	+	+	tr	4+ × 25 = 100+
	A.I.B.H.	37	1	+	tr	0	+	+	tr	3+ × 25 = 75+
	A.E.B.H.	1	10	tr	0	0	+	+	±	8+ × 25 = 200+
	A.I.B.H.	1	10	+	+	tr	+	+	±	1+ × 25 = 25+
	A.E.B.H.	37	10	+	0	0	+	+	tr	4+ × 25 = 100+
	A.I.B.H.	37	10	+	0	0	+	+	tr	4+ × 25 = 100+
Dilution 1:125	A.E.B.H.	1	1	+	+	±	+	+	±	Negative, —
	A.I.B.H.	1	1	+	+	±	+	+	±	Negative, —
	A.E.B.H.	37	1	+	+	0	+	+	tr	1+ × 125 = 125+
	A.I.B.H.	37	1	+	+	tr	+	+	tr	Negative, —
	A.E.B.H.	1	10	+	+	±	+	+	±	Negative, —
	A.I.B.H.	1	10	+	+	±	+	+	±	Negative, —
	A.E.B.H.	37	10	+	+	tr	+	+	tr	Negative, —
	A.I.B.H.	37	10	+	+	tr	+	+	tr	Negative, —
159 Dilution 1:5	A.E.B.H.	1	1	tr	0	0	+	+	tr	6+ × 5 = 30+
	A.I.B.H.	1	1	tr	0	0	+	+	tr	6+ × 5 = 30+
	A.E.B.H.	37	1	0	0	0	+	+	0	6+ × 5 = 30+
	A.I.B.H.	37	1	0	0	0	+	+	0	6+ × 5 = 30+
	A.E.B.H.	1	10	0	0	0	+	+	0	6+ × 5 = 30+
	A.I.B.H.	1	10	0	0	0	+	+	0	6+ × 5 = 30+
	A.E.B.H.	37	10	0	0	0	+	0	0	3+ × 5 = 15+
	A.E.B.H.	37	10	0	0	0	+	0	0	3+ × 5 = 15+

TABLE 7—Continued

ACETONE INSOLUBLE ANTIGEN COMPARED WITH CRUDE ALCOHOLIC EXTRACT, INCUBATION AT 1 C. AND 37 C. FOR 1 HOUR AND 10 HOURS

Number of Serum	Kind of Antigen	Incuba- tion Temper- ature, C.	Incuba- tion Time, Hours	Readings						Results
				Antigen Tubes			Control Tubes			
				1	2	3	1'	2'	3'	
Dilution 1:25	A.E.B.H.	1	1	+	tr	0	+	+	±	4+ × 25 = 100+
	A.I.B.H.	1	1	+	+	±	+	+	±	Negative, —
	A.E.B.H.	37	1	±	0	0	+	+	tr	5+ × 25 = 125+
	A.I.B.H.	37	1	tr	0	0	+	+	tr	6+ × 25 = 150+
	A.E.B.H.	1	10	0	0	0	+	+	tr	8+ × 25 = 200+
	A.I.B.H.	1	10	+	±	0	+	+	tr	2+ × 25 = 50+
	A.E.B.H.	37	10	0	0	0	+	+	0	6+ × 25 = 150+
	A.I.B.H.	37	10	0	0	0	+	+	0	6+ × 25 = 150+
Dilution 1:125	A.E.B.H.	1	1	+	+	tr	+	+	±	1+ × 125 = 125+
	A.I.B.H.	1	1	+	+	±	+	+	±	Negative, —
	A.E.B.H.	37	1	+	±	0	+	+	tr	2+ × 125 = 250+
	A.I.B.H.	37	1	+	+	0	+	+	tr	1+ × 125 = 125+
	A.E.B.H.	1	10	+	0	0	+	+	±	5+ × 125 = 625+
	A.I.B.H.	1	10	+	+	tr	+	+	±	1+ × 125 = 125+
	A.E.B.H.	37	10	+	±	0	+	+	tr	2+ × 125 = 250+
	A.I.B.H.	37	10	+	±	0	+	+	tr	2+ × 125 = 250+
160 Dilution 1:10	A.E.B.H.	1	1	0	0	0	+	+	±	10+ × 10 = 100+
	A.I.B.H.	1	1	+	0	0	+	+	±	5+ × 10 = 50+
	A.E.B.H.	37	1	0	0	0	+	+	tr	8+ × 10 = 80+
	A.I.B.H.	37	1	0	0	0	+	+	tr	8+ × 10 = 80+
	A.E.B.H.	1	10	0	0	0	+	+	tr	8+ × 10 = 80+
	A.I.B.H.	1	10	0	0	0	+	+	tr	8+ × 10 = 80+
	A.E.B.H.	37	10	0	0	0	+	+	0	6+ × 10 = 60+
	A.I.B.H.	37	10	0	0	0	+	+	0	6+ × 10 = 60+
Dilution 1:50	A.E.B.H.	1	1	+	±	0	+	+	±	3+ × 50 = 150+
	A.I.B.H.	1	1	+	+	tr	+	+	±	1+ = 50 = 50+
	A.E.B.H.	37	1	+	0	0	+	+	tr	4+ × 50 = 200+
	A.I.B.H.	37	1	+	+	0	+	+	tr	1+ × 50 = 50+
	A.E.B.H.	1	10	0	0	0	+	+	±	10+ × 50 = 500+
	A.I.B.H.	1	10	+	+	0	+	+	±	2+ × 50 = 100+
	A.E.B.H.	37	10	+	0	0	+	+	tr	4+ × 50 = 200+
	A.I.B.H.	37	10	+	±	0	+	+	tr	2+ × 50 = 100+
Dilution 1:250	A.E.B.H.	1	1	+	+	±	+	+	±	Negative, —
	A.I.B.H.	1	1	+	+	±	+	+	±	Negative, —
	A.E.B.H.	37	1	+	+	±	+	+	±	Negative, —
	A.I.B.H.	37	1	+	+	±	+	+	±	Negative, —
	A.E.B.H.	1	10	+	+	0	+	+	±	2+ × 250 = 500+
	A.I.B.H.	1	10	+	+	±	+	+	±	Negative, —
	A.E.B.H.	37	10	+	+	tr	+	+	tr	Negative, —
	A.I.B.H.	37	10	+	+	tr	+	+	tr	Negative, —

Table 7 shows the results obtained by comparing acetone insoluble antigen of beef heart with alcoholic extract of beef heart. Alcoholic extract of beef heart gave much stronger positive results than did the acetone insoluble antigen, and incubation at 1 C. gave much stronger positive results than did incubation at 37 C. With the acetone insoluble antigen incubation at 37 C. gave stronger positive results than did incubation at 1 C.

TEST 8

Alcoholic extract of beef heart (A. E. B. H.) was compared with alcoholic extract of human heart (A. E. H. H.) at 1 C. and 37 C. for 1 hour and 10 hours on 12 serums—161 to 172, inclusive.

TABLE 8

ALCOHOLIC EXTRACT OF BEEF HEART COMPARED WITH ALCOHOLIC EXTRACT OF HUMAN HEART,
INCUBATION AT 1 C. AND 37 C. FOR 1 HOUR AND 10 HOURS

Number of Serum	Kind of Antigen	Incuba- tion Temper- ature, C.	Incuba- tion Time, Hours	Readings						Results
				Antigen Tubes			Control Tubes			
				1	2	3	1'	2'	3'	
161	A.E.H.H.	1	1	+	+	tr	+	+	±	Weakly positive, 1+
	A.E.B.H.	1	1	+	+	tr	+	+	±	Weakly positive, 1+
	A.E.H.H.	37	1	+	+	0	+	+	±	Moderately positive, 2+
	A.E.B.H.	37	1	+	+	0	+	+	±	Moderately positive, 2+
	A.E.H.H.	1	10	+	0	0	+	+	±	Strongly positive, 5+
	A.E.B.H.	1	10	+	0	0	+	+	±	Strongly positive, 5+
	A.E.H.H.	37	10	+	±	0	+	+	tr	Moderately positive, 2+
	A.E.B.H.	37	10	+	±	0	+	+	tr	Moderately positive, 2+
162	A.E.H.H.	1	1	+	+	0	+	+	±	Moderately positive, 2+
	A.E.B.H.	1	1	+	+	0	+	+	±	Moderately positive, 2+
	A.E.H.H.	37	1	+	+	0	+	+	±	Moderately positive, 2+
	A.E.B.H.	37	1	+	+	0	+	+	±	Moderately positive, 2+
	A.E.H.H.	1	10	0	0	0	+	+	±	Strongly positive, 10+
	A.E.B.H.	1	10	0	0	0	+	+	±	Strongly positive, 10+
	A.E.H.H.	37	10	+	±	0	+	+	tr	Moderately positive, 2+
	A.E.B.H.	37	10	+	±	0	+	+	tr	Moderately positive, 2+
163	A.E.H.H.	1	1	+	+	tr	+	+	±	Weakly positive, 1+
	A.E.B.H.	1	1	+	+	tr	+	+	±	Weakly positive, 1+
	A.E.H.H.	37	1	+	+	0	+	+	±	Moderately positive, 2+
	A.E.B.H.	37	1	+	+	0	+	+	±	Moderately positive, 2+
	A.E.H.H.	1	10	0	0	0	+	+	±	Strongly positive, 10+
	A.E.B.H.	1	10	0	0	0	+	+	±	Strongly positive, 10+
	A.E.H.H.	37	10	+	±	0	+	+	tr	Moderately positive, 2+
	A.E.B.H.	37	10	+	±	0	+	+	tr	Moderately positive, 2+
164	A.E.H.H.	1	1	+	+	0	+	+	±	Moderately positive, 2+
	A.E.B.H.	1	1	+	+	0	+	+	±	Moderately positive, 2+
	A.E.H.H.	37	1	+	+	0	+	+	±	Moderately positive, 2+
	A.E.B.H.	37	1	+	+	0	+	+	±	Moderately positive, 2+
	A.E.H.H.	1	10	0	0	0	+	+	±	Strongly positive, 10+
	A.E.B.H.	1	10	0	0	0	+	+	±	Strongly positive, 10+
	A.E.H.H.	37	10	+	+	0	+	+	±	Moderately positive, 2+
	A.E.B.H.	37	10	+	+	0	+	+	±	Moderately positive, 2+
165	A.E.H.H.	1	1	+	+	tr	+	+	±	Weakly positive, 1+
	A.E.B.H.	1	1	+	+	tr	+	+	±	Weakly positive, 1+
	A.E.H.H.	37	1	+	+	tr	+	+	±	Weakly positive, 1+
	A.E.B.H.	37	1	+	+	tr	+	+	±	Weakly positive, 1+
	A.E.H.H.	1	10	+	0	0	+	+	±	Strongly positive, 5+
	A.E.B.H.	1	10	+	0	0	+	+	±	Strongly positive, 5+
	A.E.H.H.	37	10	+	+	tr	+	+	±	Weakly positive, 1+
	A.E.B.H.	37	10	+	+	tr	+	+	±	Weakly positive, 1+
166	A.E.H.H.	1	1	+	+	tr	+	+	±	Weakly positive, 1+
	A.E.B.H.	1	1	+	+	tr	+	+	±	Weakly positive, 1+
	A.E.H.H.	37	1	+	+	tr	+	+	±	Weakly positive, 1+
	A.E.B.H.	37	1	+	+	tr	+	+	±	Weakly positive, 1+
	A.E.H.H.	1	10	tr	0	0	+	+	±	Strongly positive, 8+
	A.E.B.H.	1	10	±	0	0	+	+	±	Strongly positive, 6+
	A.E.H.H.	37	10	+	+	tr	+	+	±	Weakly positive, 1+
	A.E.B.H.	37	10	+	+	tr	+	+	±	Weakly positive, 1+

TABLE 8—Continued

ALCOHOLIC EXTRACT OF BEEF HEART COMPARED WITH ALCOHOLIC EXTRACT OF HUMAN HEART,
INCUBATION AT 1 C. AND 37 C. FOR 1 HOUR AND 10 HOURS

Number of Serum	Kind of Antigen	Incuba- tion Temper- ature, C.	Incuba- tion Time, Hours	Readings						Results	
				Antigen Tubes			Control Tubes				
				1	2	3	1'	2'	3'		
167	A.E.H.H.	1	1	+	+	tr	+	+	±	Weakly positive,	1+
	A.E.B.H.	1	1	+	+	tr	+	+	±	Weakly positive,	1+
	A.E.H.H.	37	1	+	+	±?	+	+	±	Faintly positive,	±
	A.E.B.H.	37	1	+	+	tr	+	+	±	Weakly positive,	1+
	A.E.H.H.	1	10	0	0	0	+	+	±	Strongly positive,	10+
	A.E.B.H.	1	10	0	0	0	+	+	±	Strongly positive,	10+
	A.E.H.H.	37	10	+	+	tr	+	+	±	Weakly positive,	1+
	A.E.B.H.	37	10	+	+	0	+	+	±	Moderately positive,	2+
168	A.E.H.H.	1	1	+	+	0	+	+	±	Moderately positive,	2+
	A.E.B.H.	1	1	+	+	tr	+	+	±	Weakly positive,	1+
	A.E.H.H.	37	1	+	+	tr	+	+	±	Weakly positive,	1+
	A.E.B.H.	37	1	+	+	0	+	+	±	Moderately positive,	2+
	A.E.H.H.	1	10	tr	0	0	+	+	±	Strongly positive,	8+
	A.E.B.H.	1	10	tr	0	0	+	+	±	Strongly positive,	8+
	A.E.H.H.	37	10	+	+	tr	+	+	±	Weakly positive,	1+
	A.E.B.H.	37	10	+	+	0	+	+	±	Moderately positive,	2+
169	A.E.H.H.	1	1	+	+	0	+	+	±	Moderately positive,	2+
	A.E.B.H.	1	1	+	+	0	+	+	±	Moderately positive,	2+
	A.E.H.H.	37	1	+	+	tr	+	+	±	Weakly positive,	1+
	A.E.B.H.	37	1	+	+	0	+	+	±	Moderately positive,	2+
	A.E.H.H.	1	10	0	0	0	+	+	±	Strongly positive,	10+
	A.E.B.H.	1	10	0	0	0	+	+	±	Strongly positive,	10+
	A.E.H.H.	37	10	+	+	tr	+	+	±	Weakly positive,	1+
	A.E.B.H.	37	10	+	+	0	+	+	±	Moderately positive,	2+
170	A.E.H.H.	1	1	+	+	tr	+	+	±	Weakly positive,	1+
	A.E.B.H.	1	1	+	+	0	+	+	±	Moderately positive,	2+
	A.E.H.H.	37	1	+	+	0	+	+	±	Moderately positive,	2+
	A.E.B.H.	37	1	+	+	0	+	+	±	Moderately positive,	2+
	A.E.H.H.	1	10	0	0	0	+	+	±	Strongly positive,	10+
	A.E.B.H.	1	10	0	0	0	+	+	±	Strongly positive,	10+
	A.E.H.H.	37	10	+	+	0	+	+	±	Moderately positive,	2+
	A.E.B.H.	37	10	+	+	0	+	+	±	Moderately positive,	2+
171	A.E.H.H.	1	1	+	+	0	+	+	±	Moderately positive,	2+
	A.E.B.H.	1	1	+	+	0	+	+	±	Moderately positive,	2+
	A.E.H.H.	37	1	+	+	0	+	+	±	Moderately positive,	2+
	A.E.B.H.	37	1	+	+	0	+	+	±	Moderately positive,	2+
	A.E.H.H.	1	10	0	0	0	+	+	±	Strongly positive,	10+
	A.E.B.H.	1	10	0	0	0	+	+	±	Strongly positive,	10+
	A.E.H.H.	37	10	+	±	0	+	+	tr	Moderately positive,	2+
	A.E.B.H.	37	10	+	tr	0	+	+	tr	Strongly positive,	3+
172	A.E.H.H.	1	1	+	+	tr	+	+	±	Weakly positive,	1+
	A.E.B.H.	1	1	+	+	0	+	+	±	Moderately positive,	2+
	A.E.H.H.	37	1	+	+	0	+	+	±	Moderately positive,	2+
	A.E.B.H.	37	1	+	+	0	+	+	±	Moderately positive,	2+
	A.E.H.H.	1	10	tr	0	0	+	+	±	Strongly positive,	8+
	A.E.B.H.	1	10	0	0	0	+	+	±	Strongly positive,	10+
	A.E.H.H.	37	10	+	±	0	+	+	tr	Moderately positive,	2+
	A.E.B.H.	37	10	+	±	0	+	+	tr	Moderately positive,	2+

Table 8 shows that with very slight exceptions the results obtained with alcoholic extract of beef heart were identical with the results obtained with alcoholic extract of human heart. Slight differences are noticed with serums 166, 167, 168, 169, 171 and 172. These differences are so slight that they may be due to technical errors.

TEST 9

With Serums 173 to 184, inclusive, acetone insoluble antigen of beef heart (A. I. B. H.) was compared with alcoholic extract of human heart (A. E. H. H.) at 1 C. and 37 C. for 1 hour and 10 hours.

TABLE 9

ACETONE INSOLUBLE ANTIGEN OF BEEF HEART COMPARED WITH ALCOHOLIC EXTRACT OF HUMAN HEART, INCUBATION AT 1 C. AND 37 C. FOR 1 HOUR AND 10 HOURS

Number of Serum	Kind of Antigen	Incu- ba- tion Temper- ature, C.	Incuba- tion Time, Hours	Readings						Results	
				Antigen Tubes			Control Tubes				
				1	2	3	1'	2'	3'		
173	A.E.H.H.	1	1	+	+	±	+	+	±	Negative,	—
	A.I.B.H.	1	1	+	+	±	+	+	±	Negative,	—
	A.E.H.H.	37	1	+	+	tr	+	+	±	Weakly positive,	1+
	A.I.B.H.	37	1	+	+	±	+	+	±	Negative,	—
	A.E.H.H.	1	10	+	0	0	+	+	±	Strongly positive,	5+
	A.I.B.H.	1	10	+	+	±	+	+	±	Negative,	—
	A.E.H.H.	37	10	+	+	0	+	+	±	Moderately positive,	2+
	A.I.B.H.	37	10	+	+	tr	+	+	±	Weakly positive,	1+
174	A.E.H.H.	1	1	+	+	±?	+	+	±	Faintly positive,	±
	A.I.B.H.	1	1	+	+	±	+	+	±	Negative,	—
	A.E.H.H.	37	1	+	+	±?	+	+	±	Faintly positive,	±
	A.I.B.H.	37	1	+	+	±	+	+	±	Negative,	—
	A.E.H.H.	1	10	tr	0	0	+	+	±	Strongly positive,	8+
	A.I.B.H.	1	10	+	+	±	+	+	±	Negative,	—
	A.E.H.H.	37	10	+	+	tr	+	+	±	Weakly positive,	1+
	A.I.B.H.	37	10	+	+	±	+	+	±	Negative,	—
175	A.E.H.H.	1	1	+	+	±	+	+	±	Negative,	—
	A.I.B.H.	1	1	+	+	±	+	+	±	Negative,	—
	A.E.H.H.	37	1	+	+	±	+	+	±	Negative,	—
	A.I.B.H.	37	1	+	+	±	+	+	±	Negative,	—
	A.E.H.H.	1	10	+	tr	0	+	+	±	Strongly positive,	4+
	A.I.B.H.	1	10	+	+	±	+	+	±	Negative,	—
	A.E.H.H.	37	10	+	+	tr	+	+	±	Weakly positive,	1+
	A.I.B.H.	37	10	+	+	±?	+	+	±	Faintly positive,	±
176	A.E.H.H.	1	1	+	tr	0	+	+	±	Strongly positive,	4+
	A.I.B.H.	1	1	+	+	±	+	+	±	Negative,	—
	A.E.H.H.	37	1	+	±	0	+	+	±	Strongly positive,	3+
	A.I.B.H.	37	1	+	+	tr	+	+	±	Weakly positive,	1+
	A.E.H.H.	1	10	0	0	0	+	+	±	Strongly positive,	10+
	A.I.B.H.	1	10	+	+	±	+	+	±	Negative,	—
	A.E.H.H.	37	10	+	±	0	+	+	±	Strongly positive,	3+
	A.I.B.H.	37	10	+	+	tr	+	+	±	Weakly positive,	1+
177	A.E.H.H.	1	1	+	±	0	+	+	±	Strongly positive,	3+
	A.I.B.H.	1	1	+	+	±	+	+	±	Negative,	—
	A.E.H.H.	37	1	+	+	0	+	+	±	Moderately positive,	2+
	A.I.B.H.	37	1	+	+	±?	+	+	±	Faintly positive,	±
	A.E.H.H.	1	10	0	0	0	+	+	±	Strongly positive,	10+
	A.I.B.H.	1	10	+	+	±	+	+	±	Negative,	—
	A.E.H.H.	37	10	+	+	0	+	+	±	Moderately positive,	2+
	A.I.B.H.	37	10	+	+	tr	+	+	±	Weakly positive,	1+
178	A.E.H.H.	1	1	+	±	0	+	+	±	Strongly positive,	3+
	A.I.B.H.	1	1	+	+	±	+	+	±	Negative,	—
	A.E.H.H.	37	1	+	+	0	+	+	±	Moderately positive,	2+
	A.I.B.H.	37	1	+	+	±?	+	+	±	Faintly positive,	±
	A.E.H.H.	1	10	0	0	0	+	+	±	Strongly positive,	10+
	A.I.B.H.	1	10	+	+	±	+	+	±	Negative,	—
	A.E.H.H.	37	10	+	+	0	+	+	±	Moderately positive,	2+
	A.I.B.H.	37	10	+	+	±?	+	+	±	Faintly positive,	±

TABLE 9—Continued

ACETONE INSOLUBLE ANTIGEN OF BEEF HEART COMPARED WITH ALCOHOLIC EXTRACT OF HUMAN HEART, INCUBATION AT 1 C. AND 37 C. FOR 1 HOUR AND 10 HOURS

Number of Serum	Kind of Antigen	Incuba- tion Temper- ature, C.	Incuba- tion Time, Hours	Readings						Results
				Antigen Tubes			Control Tubes			
				1	2	3	1'	2'	3'	
179	A.E.H.H.	1	1	+	tr	0	+	+	±	Strongly positive, 4+
	A.I.B.H.	1	1	+	+	±	+	+	±	Negative, —
	A.E.H.H.	37	1	+	tr	0	+	+	±	Strongly positive, 4+
	A.I.B.H.	37	1	+	+	0	+	+	±	Moderately positive, 2+
	A.E.H.H.	1	10	0	0	0	+	+	±	Strongly positive, 10+
	A.I.B.H.	1	10	+	+	±	+	+	±	Negative, —
	A.E.H.H.	37	10	+	tr	0	+	+	±	Strongly positive, 4+
	A.I.B.H.	37	10	+	+	0	+	+	±	Moderately positive, 2+
180	A.E.H.H.	1	1	+	tr	0	+	+	±	Strongly positive, 4+
	A.I.B.H.	1	1	+	+	±	+	+	±	Negative, —
	A.E.H.H.	37	1	+	+	0	+	+	±	Moderately positive, 2+
	A.I.B.H.	37	1	+	+	tr	+	+	±	Weakly positive, 1+
	A.E.H.H.	1	10	0	0	0	+	+	±	Strongly positive, 10+
	A.I.B.H.	1	10	+	+	±	+	+	±	Negative, —
	A.E.H.H.	37	10	+	+	0	+	+	±	Moderately positive, 2+
	A.I.B.H.	37	10	+	+	tr	+	+	±	Weakly positive, 1+
181	A.E.H.H.	1	1	+	tr	0	+	+	±	Strongly positive, 4+
	A.I.B.H.	1	1	+	+	±	+	+	±	Negative, —
	A.E.H.H.	37	1	+	tr	0	+	+	±	Strongly positive, 4+
	A.I.B.H.	37	1	+	+	0	+	+	±	Moderately positive, 2+
	A.E.H.H.	1	10	0	0	0	+	+	±	Strongly positive, 10+
	A.I.B.H.	1	10	+	+	±	+	+	±	Negative, —
	A.E.H.H.	37	10	+	tr	0	+	+	±	Strongly positive, 4+
	A.I.B.H.	37	10	+	+	0	+	+	±	Moderately positive, 2+
182	A.E.H.H.	1	1	+	tr	0	+	+	±	Strongly positive, 4+
	A.I.B.H.	1	1	+	+	±	+	+	±	Negative, —
	A.E.H.H.	37	1	+	+	0	+	+	±	Moderately positive, 2+
	A.I.B.H.	37	1	+	+	tr	+	+	±	Weakly positive, 1+
	A.E.H.H.	1	10	0	0	0	+	+	±	Strongly positive, 10+
	A.I.B.H.	1	10	+	+	±	+	+	±	Negative, —
	A.E.H.H.	37	10	+	+	0	+	+	±	Moderately positive, 2+
	A.I.B.H.	37	10	+	+	0	+	+	±	Moderately positive, 2+
183	A.E.H.H.	1	1	+	tr	0	+	+	±	Strongly positive, 4+
	A.I.B.H.	1	1	+	+	±	+	+	±	Negative, —
	A.E.H.H.	37	1	+	+	0	+	+	±	Moderately positive, 2+
	A.I.B.H.	37	1	+	+	tr	+	+	±	Weakly positive, 1+
	A.E.H.H.	1	10	0	0	0	+	+	±	Strongly positive, 10+
	A.I.B.H.	1	10	+	+	±	+	+	±	Negative, —
	A.E.H.H.	37	10	+	±	0	+	+	±	Strongly positive, 3+
	A.I.B.H.	37	10	+	+	0	+	+	±	Moderately positive, 2+
184	A.E.H.H.	1	1	+	tr	0	+	+	±	Strongly positive, 4+
	A.I.B.H.	1	1	+	+	±	+	+	±	Negative, —
	A.E.H.H.	37	1	+	+	0	+	+	±	Moderately positive, 2+
	A.I.B.H.	37	1	+	+	0	+	+	±	Moderately positive, 2+
	A.E.H.H.	1	10	0	0	0	+	+	±	Strongly positive, 10+
	A.I.B.H.	1	10	+	+	±	+	+	±	Negative, —
	A.E.H.H.	37	10	+	±	0	+	+	±	Strongly positive, 3+
	A.I.B.H.	37	10	+	+	0	+	+	±	Moderately positive, 2+

Table 9 shows that alcoholic extract of human heart gave much stronger positive results than did acetone insoluble antigen of beef heart. With alcoholic extract of human heart incubation at 1 C. gave much stronger positive results than did incubation at 37 C., while with acetone insoluble antigen stronger positive results were obtained at 37 C. than at 1 C.

TEST 10

When sensitized blood corpuscles are added to serum-complement-antigen mixture immediately after the latter has been removed from the water-bath at 1 C. and the whole mixture then placed in the incubator at 37 C. hemolysis does not take place until the contents of the tubes have become warm, while agglutination of the corpuscles is rapid even at a temperature of 1 C. In order to determine whether or not the mixture may be warmed before the sensitized blood corpuscles are added each of 16 serums (185 to 200, inclusive) was divided into two portions, A and B. Portion A was left in the water-bath at 1 C. for 10 hours and the sensitized blood corpuscles were added immediately after the tubes had been removed from the cold water-bath. Portion B was left in the cold water-bath for 9.5 hours, then was placed in the incubator at 37 C. for 0.5 hour before the sensitized blood corpuscles were added.

TABLE 10

NINE AND ONE-HALF HOUR INCUBATION AT 1 C. + ONE-HALF HOUR AT 37 C. COMPARED WITH TEN-HOUR INCUBATION AT 1 C.

Number of Serum	Portion	Incu- ba- tion Temper- ature, C.	Incuba- tion Time, Hours	Readings						Results	
				Antigen Tubes			Control Tubes				
				1	2	3	1'	2'	3'		
185	A	1	10	+	tr	0	+	+	±	Strongly positive,	4+
	B	1	9.5	+	tr	0	+	+	±	Strongly positive,	4+
186	A	1	10	±	0	0	+	+	±	Strongly positive,	6+
	B	1	9.5	±	0	0	+	+	±	Strongly positive,	6+
187	A	1	10	tr	0	0	+	+	±	Strongly positive,	8+
	B	1	9.5	tr	0	0	+	+	±	Strongly positive,	8+
188	A	1	10	±	0	0	+	+	±	Strongly positive,	6+
	B	1	9.5	±	0	0	+	+	±	Strongly positive,	6+
189	A	1	10	+	tr	0	+	+	±	Strongly positive,	4+
	B	1	9.5	+	tr	0	+	+	±	Strongly positive,	4+
190	A	1	10	+	0	0	+	+	±	Strongly positive,	5+
	B	1	9.5	+	0	0	+	+	±	Strongly positive,	5+
191	A	1	10	±	0	0	+	+	±	Strongly positive,	6+
	B	1	9.5	+	0	0	+	+	±	Strongly positive,	5+
192	A	1	10	+	tr	0	+	+	±	Strongly positive,	4+
	B	1	9.5	+	tr	0	+	+	±	Strongly positive,	4+
193	A	1	10	±	0	0	+	+	±	Strongly positive,	6+
	B	1	9.5	±	0	0	+	+	±	Strongly positive,	6+
194	A	1	10	+	±	0	+	+	±	Strongly positive,	3+
	B	1	9.5	+	±	0	+	+	±	Strongly positive,	3+
195	A	1	10	+	+	tr	+	+	±	Weakly positive,	1+
	B	1	9.5	+	+	tr	+	+	±	Weakly positive,	1+
196	A	1	10	+	+	0	+	+	±	Moderately positive,	2+
	B	1	9.5	+	+	0	+	+	±	Moderately positive,	2+
197	A	1	10	+	±	0	+	+	±	Strongly positive,	3+
	B	1	9.5	+	±	0	+	+	±	Strongly positive,	3+
198	A	1	10	+	0	0	+	+	±	Strongly positive,	5+
	B	1	9.5	+	0	0	+	+	±	Strongly positive,	5+
199	A	1	10	+	±	0	+	+	±	Strongly positive,	3+
	B	1	9.5	+	±	0	+	+	±	Strongly positive,	3+
200	A	1	10	+	tr	0	+	+	±	Strongly positive,	4+
	B	1	9.5	+	tr	0	+	+	±	Strongly positive,	4+

Table 10 shows that portion B gave results that were identical with those given by portion A. In portion A the corpuscles were badly agglutinated before hemolysis took place; the tubes had to be frequently shaken and hemolysis was very slow, while in portion B hemolysis was rapid and with little or no agglutination of the blood corpuscles.

SUMMARY AND CONCLUSIONS

Incubation at 1 C. gave stronger positive results than did incubation at 2 C., 3 C., 4 C., 5 C., 6 C., 7 C., 8 C., 9 C., 10 C., or 37 C., while the results obtained at 0.5 C. were identical with those obtained at 1 C.

Incubation for 24 hours at 1 C. gave stronger positive results than incubation at 1 C. for 15 hours; incubation for 15 hours at 1 C. gave stronger positive results than did incubation for 10 hours at 1 C.; incubation for 10 hours at 1 C. gave stronger positive results than did incubation for 5 hours at 1 C., and incubation for 5 hours at 1 C. gave stronger positive results than did incubation for 1 hour at 1 C.

Incubation for 10 hours at 1 C. gave uniformly good results, while with one lot of complement 15-hour incubation at 1 C. and 24-hour incubation at 1 C. gave bad results. At the present time 10-hour incubation at 1 C. is the longest incubation time which has met all requirements.

Twenty-four serums from healthy persons gave negative results under 10-hour incubation at 1 C. and under 15-hour incubation at 1 C.

Alcoholic extract of beef heart gave much stronger positive results than did acetone insoluble antigen of beef heart. The alcoholic extract gave the strong positive results at 1 C., while the acetone insoluble antigen gave stronger positive results at 37 C. than at 1 C.

At 1 C. alcoholic extract of beef heart gave results that were identical with the results given by alcoholic extract of human heart at 1 C.

Acetone insoluble antigen of beef heart gave much weaker positive results than did alcoholic extract of human heart.

Warming the serum-complement-antigen mixture before the sensitized blood corpuscles were added gave the same results as when the sensitized blood corpuscles were added to the cold serum-complement-antigen mixture. Warming the serum-complement-antigen mixture before the sensitized blood corpuscles are added is a decided advantage because hemolysis is much more rapid and agglutination of the corpuscles is much less.

THE PROTEUS GROUP OF ORGANISMS WITH SPECIAL REFERENCE TO AGGLUTINATION AND FER- MENTATION REACTIONS AND TO CLASSIFICATION

IDA A. BENGTON

*From the Department of Hygiene and Bacteriology of the University of Chicago, and Hygienic
Laboratory of the U. S. Public Health Service*

INTRODUCTION

The proteus group, of which *Proteus vulgaris* may be considered the type species, is frequently referred to in the literature, although its members are often indefinitely characterized. The term "proteus group" has been loosely used to include a number of organisms which are distantly related.

Members of the proteus group are of interest on account of their occurrence under widely different conditions. They are usually thought of as saprophytic in nature, but the list of pathologic processes in which they are present, occasionally as the primary cause of disease and frequently as secondary agents enumerated by Meyerhof, justifies their characterization by this author as facultative parasites.

The recent work of certain French authors, including the studies of Metchnikoff and Berthelot on the relation of *Proteus vulgaris* to infantile diarrhea, and later that of Horowitz, who describes this organism as the causal agent in an epidemic of gastro-enteritis simulating dysentery, as well as the accounts of food poisoning epidemics attributed to this organism, are of special interest.

The rôle of *Proteus vulgaris* as a secondary agent in pathological processes in which admittedly pathogenic organisms are concerned is of importance. In the literature an increase in the virulence of *B. diphtheriae* associated with *Proteus vulgaris* has been noted. The presence of *Proteus vulgaris* in infections with pneumococcus, streptococcus and staphylococcus has been observed by several authors. It has been shown experimentally that the virulence of cultures of these cocci may be increased in the presence of *Proteus vulgaris* or its metabolic products.

Proteus vulgaris (associated with streptococci, *B. coli*, *B. lactis-aerogenes*, *B. welchii*, and other organisms) has been frequently observed as an accompanying agent in wound infections. Swan and Goadby in their recent work in vaccine therapy in septic gunshot wounds recommend that cases of septic wounds receive an initial dose of a polyvalent vaccine of streptococcus and proteus strains. The rôle of *Proteus vulgaris* and other organisms in symbiosis with pathogenic anaerobes is worthy of consideration. In this connection Douglas, Fleming and Colebrook have recently determined experimentally that *B. perfringens*, *B. edematis maligni* and *B. hibler* in association with *Proteus vulgaris* and other aerobes commonly occurring in wounds multiply much more rapidly than when alone.

The Weil-Felix reaction in the diagnosis of typhus exanthematicus is a recent development. While the organism is not connected etiologically with this disease, it has been found that the serum of patients suffering from this disease agglutinates regularly certain strains of *Proteus vulgaris* originally isolated by Weil and Felix from the urine and stools of such patients, in dilutions of 1:100 to 1:2000 or higher.

These facts justify a study of the proteus group, from the standpoint of classification, since many widely different organisms have been classified here, and also from the standpoint of pathogenicity. The present paper embodies the results obtained in a study of the cultural and agglutination properties of a number of organisms isolated from various sources. An attempt has been made to determine the characteristics of the group as a whole as well as the differential characters of the various members, by a review of the more important literature as well as by the study of a limited number of cultures.

The cultures studied include those shown in Table 1.

All cultures were planted in plain broth three successive times, then plated on gelatin or Endo medium and colonies fished and subjected to preliminary tests consisting of planting in dextrose, lactose and saccharose broth fermentation tubes and in gelatin. In most cases several cultures from the same source were studied, and if their behavior was uniform in the various mediums used, the duplicate cultures were discarded and the results not included.

In the following discussion, all of the cultures included in Table 1, with the exception of the two designated as *B. zopfii* and *B. proteus zenkeri*, and the cultures of *Pseudomonas protea*, will be considered first as a whole. While not all of the cultures correspond to *Proteus vulgaris* it has been found convenient to discuss them together, pointing out differences as the occasion arises. The discussion of the literature in this section will in general refer to the *vulgaris* type.

TABLE 1
DESCRIPTION OF CULTURES STUDIED

No. of Culture	Source
	(a) Feces
{11	
{13	Typhoid feces 53-3
{17	
{18	Typhoid feces 601
20	Typhoid feces 611
{29	Feces in food poisoning epidemic case, tho not proven to be causal agent.
{32	
{54	Old typhoid feces B5 (examination of feces for typhoid carriers)
{55	
68	Culture from child's stool, Northwestern University Medical School
96	Normal feces 41HT
98	Normal feces Fe. 106
99	Normal feces Fe. 100
103	Normal feces 48HT
108	Normal feces Fe. 97
115	Normal feces Fe. 93
	(b) Meat
{34	Chopped meat
{35	
{36	
{43	Chopped meat
{45	
63	Chopped meat
78	Putrifying meat, Sheffield Scientific School
113	Chopped meat
114	Sausage
	(c) Water
{24	Water (filtered ?)
{27	
79	Stagnant water. Sheffield Scientific School
110	Sewage 7634
111	Sewage 7634
112	Sewage 7641
	(d) Necropsied animals
51	Peritoneal fluid. Guinea-pig.
53	Peritoneal fluid. Guinea-pig
94	Heart's blood. Mouse
	(e) Blood
69	Blood culture of H.
	(f) Air ?
{47	Contamination of B. coli growing in phenol solution
{49	
	(g) Wound bandage
75	Wound bandage, Sheffield Scientific School
	(h) Saliva
76	Dog's saliva, Sheffield Scientific School
	(i) Laboratory cultures from various sources and cultures of unknown origin
1	Proteus vulgaris I (University of Chicago)
2	Proteus vulgaris II (University of Chicago)
3	Proteus vulgaris Kral (University of Chicago)
4	Proteus mirabilis (University of Chicago)
64	Proteus vulgaris (University of Minnesota)
65	Proteus vulgaris (University of Minnesota)
67	Proteus vulgaris (University of Minnesota)
70	226 B. proteus, laboratory culture, American Museum Natural History
71	142 B. proteus vulgaris, laboratory culture, American Museum of Natural History
77	Proteus vulgaris, University of Pennsylvania. Sheffield Scientific School
80	Proteus vulgaris, Novy, Mich. Sheffield Scientific School
92	B. proteus vulgaris. A. I. K.
	(j) Proteus zopfii and zenkeri
73	B. proteus zopfii, laboratory culture, American Museum of Natural History
85	B. zenkeri, laboratory culture, Hygienic Laboratory
	(k) Pseudomonas protea
86	Ps. protea 362t. Hygienic Laboratory. Isolated from filtered water (W. H. Frost)
87	Ps. protea 366. Hygienic Laboratory. Isolated from filtered water (W. H. Frost)

METHODS OF ISOLATION

A number of different methods have been recommended for the isolation of members of the proteus group. In my work cultures were isolated by several of the methods described below, the majority however, by growing in dextrose broth fermentation tubes, plating in gelatin or streaking on agar or Endo plates, and fishing characteristic colonies. Direct streaking on Endo medium was also used in the isolation of *Proteus* strains from normal stools.

Metchnikoff and Bertrand used the method of Choukévitch in the isolation of *Proteus vulgaris* from stools. This consisted in planting the material on the surface of tubes of gelatin, retaining at a temperature of 22 C. for one day, and if liquefied, transplanting a loopful to the condensation water of slant agar tubes. If *Proteus vulgaris* is present it grows quickly to the top of the slant, and by making several successive transfers, a pure culture may be obtained. Jordan, in a study of bacteria from river water, isolated proteus strains by the gelatin plate method and also by planting in dextrose broth fermentation tubes and then plating.

Mediums containing lactose have been used by several authors, including Feltz and Horowitz. The former used litmus lactose agar plates in isolating *Proteus vulgaris* from stools, fishing colonies which did not give a characteristic *B. coli* reaction into peptone water, and then testing this for indol, as he considered a positive indol test particularly characteristic of *Proteus vulgaris*. Horowitz, in part of his work, used a gelatin litmus lactose medium. He also used a preliminary enrichment medium, consisting of broth or peptone water and bile, after which he plated on solid mediums. Drigalski-Conradi medium has also been used by several authors.

MORPHOLOGY

The morphology of the cultures studied was determined from 24-hour-old cultures grown on agar slants at 37 C. A small amount of the drier portion of the cultures was transferred to a drop of salt solution on a slide, which after drying and fixing was stained with methyl violet for one minute. All were rods, which in general were small, varying from 0.3-0.5 by 0.8-3 mikrons. *Pseudomonas* cultures were somewhat larger, while the *B. zopfii* cultures showed organisms ranging from 4 mikrons to long filaments. The pleomorphic forms described by a number of authors were not present in all of the cultures. Certain of the cultures, however, especially those of more recent isolation showed this characteristic, some exhibiting gradations from coccoid forms to bacilli, 5-8 mikrons in length. Swollen forms were present in only one or two instances. Hauser¹ considered the pleomorphic feature of this organism to be one of its distinguishing characteristics, and Feltz also states that it is one of the most polymorphic of all organisms. Spiral forms are described by this author. The occurrence of the organism in pairs and chains including up to 20 elements has been noted by several authors. Of the cultures in this study, Nos. 78, 96, 98, 103 showed long chains of strepto-bacilli. Pfuhl notes the fact that no involution forms were present in cultures 4 months old which he examined.

PRESENCE OF FLAGELLA AND SPORES

Flagella.—The presence of peritrichic flagella is generally conceded to be characteristic of *Proteus vulgaris*. Silberschmidt describes long flagella all around the bacillus, of which there were 4, 8 12 or more. Wesenberg found up to 20 or more flagella in cultures of *Proteus vulgaris* isolated in an epidemic

TABLE 2

CULTURAL CHARACTERISTICS

No. of Culture	Rods > 1.5 μ	Pleo- mor- phism	Mo- tility	Rapidly Spread- ing Growth on Agar	Pigment Production in Broth	Gelatin Liquefac- tion		Brom-cresol-purple Milk			Indol	Reduction of Nitrates		Voges- kauer Reac- tion	Gas Production			
						3 da.	30 da.	Acid	Coag.	Pep.		Alk.	Ni- trates		Am- monia	Dex- trose Broth	Lac- tose Broth	Saccha- rose Broth
Proteus vulgaris																		
Feces 11.....	—	—	+	+	+	+	+	+	+	+	+	+	+	—	+	+	—	+
17.....	—	—	+	+	+	+	+	+	+	+	—	+	—	—	+	+	—	+
29.....	—	—	+	+	+	+	+	+	+	+	—	+	—	—	+	+	—	+
55.....	—	—	+	+	+	+	+	+	+	+	—	+	—	—	+	+	—	+
96.....	—	+	+	+	+	+	+	+	+	+	—	+	—	—	+	+	—	+
98.....	—	—	+	+	+	+	+	+	+	+	—	+	—	—	+	+	—	+
99.....	—	—	+	+	+	+	+	+	+	+	—	+	—	—	+	+	—	+
103.....	—	—	+	+	+	+	+	+	+	+	—	+	—	—	+	+	—	+
108.....	—	+	+	+	+	+	+	+	+	+	—	+	—	—	+	+	—	+
115.....	—	+	+	+	+	+	+	+	+	+	—	+	—	—	+	+	—	+
Meat 34.....	—	—	+	+	—	+	+	+	+	+	+	+	+	—	+	+	—	+
78.....	+	+	+	+	+	+	+	+	+	+	+	+	+	—	+	+	—	+
113.....	+	+	+	+	+	+	+	+	+	+	+	+	+	—	+	+	—	+
114.....	+	+	+	+	+	+	+	+	+	+	+	+	+	—	+	+	—	+
Water 79.....	—	—	+	+	+	+	+	+	+	+	+	+	+	—	+	+	—	+
110.....	—	+	+	+	+	+	+	+	+	+	+	+	+	—	+	+	—	+
111.....	+	+	+	+	+	+	+	+	+	+	—	+	—	—	+	+	—	+
112.....	+	+	+	+	+	+	+	+	+	+	—	+	—	—	+	+	—	+
Neeropsied animals																		
53.....	+	+	+	+	+	+	+	+	+	+	+	+	+	—	+	+	—	+
94.....	+	+	+	+	+	+	+	+	+	+	+	+	+	—	+	+	—	+
Wound bandage 75	+	—	+	+	+	+	+	+	+	+	—	+	—	—	+	+	—	+
Dog's saliva 76.....	—	—	+	+	+	+	+	+	+	+	—	+	—	—	+	+	—	+

of meat poisoning. Pfuhl, Meyerhof, Cantu and Levy also demonstrated numerous flagella. Frost, who isolated an organism from filtered water, which he designated as *Pseudomonas protea*, calls attention to the fact that the descriptions of *Proteus vulgaris* cited in the literature are not sufficient to differentiate between it and the organism which he isolated. In my work several cultures of *Ps. protea* isolated by Frost have been included, in order to determine by cultural and agglutination reactions their relation to *Proteus vulgaris*.

Culture 4 in this study, which was typical in all respects of *Proteus vulgaris*, when stained by the Loeffler method showed numerous flagella surrounding the entire organism, which in comparison with the typhoid bacillus were much more numerous and much finer. Culture 24 showed polar flagella corresponding to *Ps. protea* as described by Frost.

Spores.—The absence of spores has been demonstrated by a number of authors and is generally accepted as characteristic of *Proteus vulgaris*.

GRAM STAIN

There is some disagreement among various authors in regard to the gram staining properties of *Proteus vulgaris*, but the consensus of opinion is in favor of a gram-negative stain. Feltz states that fresh cultures stained with anilin gentian violet made up at the time of using are always gram-positive. Horowitz found results with the Gram stain not always clear cut, but the majority of organisms gram-negative. Van Loghem also states that in young cultures, there are many gram-positive forms, but that in 24-hour-old cultures they are mostly gram-negative. The majority of authors, however, describe *proteus* as gram-negative. It is probable that some of the earlier authors who described gram-positive cultures were not describing *Proteus vulgaris* forms.

The following technic was used in determining the gram-staining properties of the cultures in this study. Smears were made from 24-hour-old agar slants and stained as follows:

1. Fixed by heat.
2. Methyl violet applied 1 minute, and shaken off slide.
3. Gram's iodine solution applied 1 minute and shaken off slide.
4. Slide rinsed with absolute alcohol until no color was observable in the rinsings.
5. Washed with tap water.
6. Stained with 0.1% basic fuchsin 10 seconds.

By this method all the cultures were found to be gram-negative (with the exception of the *zopfii* and *zenkeri* strains).

MOTILITY

Motility was studied by the usual hanging drop method, using 24-hour cultures from agar slants, grown at incubator temperature, a small amount of the growth being transferred to a drop of salt solution on a cover-slip. The various cultures showed motility in varying degrees, and not all could be described as exhibiting active motility. The movement in most cases was both rotatory and progressive, in a zigzag direction. One of the cultures under *proteus* species, No. 63, and the cultures *B. zopfii* showed only slight motility.

Cantu found motility to be at a maximum in 12-40 hours at incubator temperature, after which time it diminished, so that in 3 days the organisms were almost immotile.

GROWTH ON AGAR

A characteristic of *Proteus vulgaris* not emphasized by some authors as much as it deserves, is the distinctive growth of the organism on agar slants, namely, its tendency to spread very rapidly. This has been observed by French authors and has been taken advantage of in its isolation from mixed cultures. Metchnikoff, who studied 204 cultures from stools, found that only five failed to rise rapidly to the top of the slant when planted in the lower part of the tube.

In this study one culture (92) which was typical in other respects failed to reach the top of the tube in from 6 to 24 hours when planted at the bottom of the slant. The growth of cultures of *Proteus vulgaris* may be described as effuse, moist and moderately luxuriant.

None of the cultures included under the headings *Proteus* species, *B. cloacae*, *B. zopfii*, and *Pseudomonas protea* showed the characteristic of rapidly spreading growth of *Proteus vulgaris*.

PIGMENT PRODUCTION

The production of brown pigment is considered by some authors to be characteristic of *proteus* strains. The presence of a brown coloration was observed by Jordan in the case of strains of *Proteus* varieties isolated from river water.

Pigment production in cultures of *Proteus vulgaris* appears to be as much an effect in the medium as in the growth itself. It is therefore evident that observations on the pigment production of the surface growth on agar slants may not be a true index of the pigment producing powers of cultures. A distinct difference of color in broth cultures of typical *Proteus vulgaris* strains and other strains was noticed, and it was determined to make tests of pigment production, by growing the cultures in plain broth for 14 days and noting change of color. The tests were made by removing a sufficient amount of the culture to a flat bottomed vial of a diameter of about 1 cm., so that a layer 1 cm. in depth was obtained, placing this on a white background, and comparing with the color standards (Ridgway's) by looking downward through the fluid. A culture which had not perceptibly darkened the medium in comparison with an uninoculated tube was used as a control, as readings of the tubes showing pigment production were made more easily in comparison with a turbid than a clear medium.

The cultures which were typical in all respects, with one or two exceptions, produced pigment varying from light cadmium to antique brown (19YO-Y to 170-Yk). The control culture was recorded as straw yellow (21'0-YYd).

Cultures 34 and 78 from meat which were typical of *Proteus vulgaris* in cultural and agglutination reactions produced no pigment. Cultures in this study which are classified as *Proteus* species, *B. cloacae* and *Ps. protea*, in general produced no pigment, or very slight. These include 20 and 68 (feces), 45, 63, 24, 86, 87 (water), 51 (necropsied animal), 49 (air).

BROTH

Growth in broth showed nothing characteristic except the production of pigment as noted above. A turbid growth without pellicle formation was produced in 24 hours. At the end of 7 days, all of the cultures showed more or less precipitate at the bottom of the tube, and some a slight pellicle.

GELATIN

The power of liquefying gelatin has usually been considered to be one of the most characteristic properties of the *proteus* group. Hauser's original classification of the group into three species was based on differences in

liquefying power, the *vulgaris* type liquefying gelatin rapidly, *mirabilis* more slowly and *zenkeri* not at all. This classification was withdrawn by Hauser on observing that *zenkeri* did sometimes liquefy gelatin after long cultivation, and the two types, *mirabilis* and *zenkeri*, were considered by this author to be attenuated forms of *vulgaris*. It has been shown by several authors that the power of liquefying gelatin varies in the same culture with growth on artificial mediums. Heim states that the property of liquefying gelatin may be lost and later return.

In the cultures studied, liquefaction of gelatin was determined by inoculating the surface of gelatin stabs, and noting the amount of liquefaction in millimeters after periods of 3 days and 30 days. All of the cultures corresponding to typical *Proteus vulgaris*, with one exception, No. 71, liquefied gelatin in 3 days, and showed liquefaction varying from 10-35 mm. in 30 days. Culture 71 was retained for a longer period and in 2 months was liquefied to a depth of 15 mm. This culture was typical in all other respects, furnishing evidence that delay in the liquefaction of gelatin should not necessarily be considered a sufficient reason for classifying in different species providing the culture is otherwise typical. Cultures 20 under *Proteus* species, 68 (feces) and 51 (necropsied animal) under *B. cloacae* did not liquefy in 3 days, but showed a certain amount of liquefaction in 30 days. Cultures 45, 63 (meat), 69 (blood), 47, 49 (air), differing in certain other respects from typical *Proteus vulgaris*, liquefied gelatin promptly.

GELATIN COLONIES

The appearance of the colonies of *Proteus vulgaris* on gelatin plates is a matter to which considerable attention has been paid. "Swarming" as a criterion for classification in the *proteus* group, has been emphasized by a number of authors. Swarm colonies have been described by Hauser, Pfuhl, Weber, Silberschmidt and others. That this property is not constant, however, has been admitted by Hauser and others. Herter and Ten Broeck in a critical study of two strains of *Proteus vulgaris* state that swarming was observed in only one instance, and that in a culture which had been rapidly passed through milk tubes and then placed on glucose gelatin. Five per cent. gelatin which is recommended as the best medium for the formation of swarm colonies, however, was not used by this author.

Berthelot and Horowitz do not emphasize characteristic colonies on gelatin. Horowitz found that characteristic colonies, with tortuous prolongations were sometimes produced on gelatin plates and at other times the colonies were simply round, and that the same strain often showed both kinds of colonies. The best results were obtained by slowly cooling the plates.

It is probably true that characteristic colonies are often produced by strains when freshly isolated, but that cultures soon lose this property. Boehnke, who studied the subject of swarm colonies in detail, at the instigation of Hauser, attempted to determine under what conditions swarming takes place. He points out that certain authors have not been justified in classifying organisms in the *proteus* group, inasmuch as swarming has not been described. He states that "swarming" is the only sure criterion for *proteus*, and that if one observes threadlike prolongations and islands in active motion under the microscope, or corkscrew projections extending outward from the colony in 5% gelatin medium it is a certain demonstration of the organism. He adds, however, that this property is inconstant, and sought to explain this discrepancy by growing cultures under different conditions of temperature, concentration of medium and difference of oxygen tension, but was not able to determine the favorable conditions.

In view of the observations of the above investigators, it appears that the property of forming swarm colonies may identify the organism in question as a member of the proteus group, but nonformation of swarm colonies does not necessarily exclude an organism from the proteus group, if it is typical in other respects.

MILK

The behavior of *Proteus vulgaris* in litmus milk has been variously described by different authors. Jordan notes acid reaction followed by curdling and digestion of casein. Cantu, Wesenbery, Weber and others report preliminary acid reaction followed by curdling, digestion of casein and alkaline reaction in typical cultures. Larson and Bell¹ and Berthelot do not observe change of reaction, but found milk curdled and then peptonized. Horowitz reports that cultures coagulated milk readily at the end of 2 days, without formation of acid, which was followed by pronounced peptonization. Archibald describes acid formation without curdling, followed by an alkaline reaction 3 days later. Herter and Ten Broeck found that one culture showed no visible change in 12 days, at which time the reaction was + 1.5. In the other culture, a soft curd was present in 3 days, which showed signs of digestion. Cantu calls attention to the fact that *Proteus vulgaris* derived from animal sources coagulates milk more strongly than strains derived from other sources.

It is probable that the difference of expression of results obtained by various workers is partly due to the fact that cultures of *Proteus vulgaris* actively reduce litmus, and it is difficult to determine reaction. In order to overcome this difficulty I decided in my work to substitute for litmus milk, milk containing brom-cresol-purple as recommended by Clark and Lubs.* With this medium it was possible to distinguish readily changes in reaction, and at the end of 14 days the differences in the typical and nontypical forms were very marked.

In 24 hours all of the *Proteus vulgaris* cultures (except 71) including 11, 17, 29, 55, 96, 98, 99, 103, 108, 115 (feces), 34, 78, 113, 114 (meat), 79, 110, 111, 112 (water), 53, 94, (necropsied animals), 75 (wound bandage), 76 (dog's saliva), 1-4, 64, 65, 67, 70, 77, 92 (laboratory cultures) were slightly acid, but showed no curdling. The majority of these showed curdling in 3 days, and beginning peptonization. Nos. 67, 94, 110 and 115 showed an alkaline reaction without curdling in 3 days, but in 7 days showed curdling with partial peptonization. Culture 71 was markedly acid in 24 hours, then became decidedly alkaline and showed no peptonization in 14 days.

Of the remaining cultures, included under proteus species, and *B. cloacae*, Nos. 68 (feces), 46, 63 (meat), 47, 49 (air), 51 (necropsied animal), 69 (blood), 80 (laboratory culture) were acid at the end of 24 hours, and in the case of Cultures 45, 47, 49, sufficient acid was produced to cause coagulation of the casein. These cultures showed increased acidity in 3 days with coagulation in all except 68.

Culture 20 (feces) was alkaline in 24 hours and showed increased alkalinity in 3 days.

In 14 days the *Proteus vulgaris* cultures all showed a purple color (alkaline reaction) while the remaining cultures were a decided yellow, with hard curd (except 20, alkaline).

* The medium is made by adding 0.005% of the sodium salt of dibromo-ortho-cresol-sulfonphthalein to milk and sterilizing for 20 minutes at a pressure of 15 lbs. (Clark and Lubs: Jour. Agr. Research, 1917, 10, p. 105.)

The behavior in brom-cresol-purple milk is of value in the identification of *Proteus vulgaris*, correlating with rapidly spreading growth on agar, gelatin liquefaction, fermentation and agglutination tests.

The reaction of the cultures of *Ps. protea* in brom-cresol-purple milk contrasted strongly with that of typical *Proteus vulgaris*. More acid was produced in 24 hours, and a hard curd was produced in 3 days, which remained undigested with a strongly acid reaction at the end of 14 days.

Endo Plates.—Tests were made by streaking broth cultures on Endo plates to determine the appearance of colonies on this medium. The appearance was typical in most cases in the *Proteus vulgaris* cultures. A pink or reddish spreading colony was produced in most cases in 24 hours, but in a few cases spreading was not evident until after 48 hours, the colony first appearing as round, raised and moist. In some cases spreading and nonspreading colonies were present on the same plate, particularly if the colonies were numerous. Several cultures, including 3, 65, 77, 78, and 92, failed to show spreading in 48 hours, the colonies being simply round.

None of the cultures classed as proteus species, *B. cloacae* or *Pseudomonas protea* showed the spreading growth characteristic of *Proteus vulgaris*. Cultures 43 and 80 showed colonies with the metallic luster characteristic of *B. coli*.

FERMENTATION REACTIONS

The use of fermentation reactions in various carbohydrates and related substances has been extensively applied in the classification of certain groups of organisms, particularly the *B. coli* and paratyphoid groups and the streptococci. To a certain extent, correlation of fermentation reactions with source and pathogenicity has been demonstrated, but there are also many discrepancies in such correlations.

In rating the value of fermentation reactions as a basis for classification certain factors have to be taken into consideration. The kind of medium, the age of the cultures, which involves variations, and the length of the incubation period are of importance.

In this work it has been found that the presence or absence of meat extract in the carbohydrate medium influences to a great extent carbohydrate metabolism, particularly in the case of saccharose. Most authors describe incompletely or not at all the composition of the mediums used in the study of fermentation reactions. Presumably most of these are broth mediums containing a certain percentage of the carbohydrate.

Berthelot used as a medium peptone solution containing the various carbohydrates, in the proportion of 3% of the total volume. Tubes of peptone solution without carbohydrate were inoculated with the cultures as controls, the test for acid production being made by adding litmus solution to both carbohydrate and control tubes at the end of a certain period of incubation, and comparing results in the two tubes. The advantage of this method obviously lies in the fact that reduction of the litmus is avoided, which interferes with determination of acid production.

The age of the culture in the case of *Proteus vulgaris* apparently is involved in the problem of carbohydrate metabolism. A number of instances are quoted in the literature of variation in carbohydrate metabolism by cultures which had been kept under cultivation for a certain length of time. Theobald Smith¹ considered fermentation reactions in dextrose, saccharose and lactose more stable than other cultural reactions of *Proteus vulgaris*. In the case of the disaccharid maltose, however, several well authenticated cases of the loss of

power to ferment this sugar by cultures which originally did so are recorded. Horowitz found that cultures which at the time of isolation fermented maltose, failed to do so after having been retained in broth for one month. Thjtta also found that cultures from diarrheal stools, which originally fermented dextrose, saccharose, maltose and mannite, failed to ferment maltose and mannite in 1½ months, and fermented dextrose and saccharose in a less degree.

The period of incubation is a factor involved in the study of carbohydrate fermentations. Action on certain carbohydrates by *Proteus vulgaris* cultures is prompt, particularly in the case of dextrose, while in the case of others, such as saccharose, action may be very much delayed. This may partially account for variable results obtained with this carbohydrate by different authors.

The cultures made use of in my study were subjected to preliminary tests in dextrose, lactose and saccharose broth and for liquefaction of gelatin, and in general those cultures included which fermented dextrose and saccharose and which liquefied gelatin. This was in accordance with the suggestions of Jordan, Theobald Smith, Herter and Ten Broeck and others.

Theobald Smith, who studied the fermentation reactions of several strains of proteus, found that cultures kept under cultivation for a year showed a diminished or complete loss of power of liquefying gelatin, while the fermentation reactions in dextrose, lactose and saccharose remained the same. He therefore recommends the fermentation tube test in classifying organisms in this group, considering as proteus those varieties of organisms which ferment dextrose and saccharose but not lactose.

Jordan in a study of bacteria found in river water has included in the proteus group, organisms which ferment dextrose and saccharose, and rarely lactose, which are actively proteolytic, liquefying gelatin, and blood serum and precipitating, then digesting casein. Three subdivisions are considered in the group: (1) *Proteus vulgaris*; (2) proteus varieties; (3) *B. cloacae*.

Herter and Ten Broeck who studied two proteus strains, one of which was derived from putrid material, found that the only constant cultural properties exhibited by the two cultures were in the matter of fermentation reactions in dextrose, lactose and saccharose, though the two cultures produced the same chemical products and their pathogenic properties were the same.

In my study the amount of gas present in dextrose and saccharose broth fermentation tubes (Smith tubes) was recorded in these preliminary tests. Gas was nearly always present in dextrose in 24 hours, and in 7 days, the amount varied from 15-40% in the *Proteus vulgaris* cultures, the majority showing about 30% of gas. The cultures classified as proteus species varied from 3-50% in gas production. Two cultures classified as *B. cloacae* produced 65-80% of gas and another produced 5%.

Production of gas in saccharose broth was delayed and was considerably less in amount than in dextrose broth. Most of the *Proteus vulgaris* cultures showed a very small amount or no gas in two days, and not above 15% in 7 days. Culture 94, typical in other respects, produced no gas in saccharose broth in 14 days. The cultures classified as proteus species in general produced small amounts of gas in saccharose broth, and *B. cloacae* large amounts.

Most authors are in accord regarding fermentation of dextrose and non-fermentation of lactose by *Proteus vulgaris*, but fermentation of saccharose has given variable results in the hands of different workers. Berthelot, who studied a number of strains of *Proteus vulgaris* derived for the most part from cases of infantile diarrhea, and Horowitz who studied strains isolated from feces in an epidemic of gastro-enteritis found the reaction variable in saccharose mediums. Larson and Bell,¹ observing a number of strains derived

from pathologic sources noted that saccharose was not acted on by most of the strains. Bahr and Thomsen, in a study of proteus strains isolated from feces, classified them in three types, two of which produced acid and gas in saccharose and one which did not—the majority of the cultures, however, belonging to the first class. Archibald describes a strain isolated from a case of choleraic diarrhea, which had no action on saccharose.

Tests on the cultures studied in my work were also made by the hydrogen-ion concentration method, using the following: Monosaccharid, dextrose; disaccharids, lactose, saccharose and maltose; trisaccharid, raffinose; and alcohol, mannite (Table 3).

The medium adopted was one without meat extract and consisted of peptone water (1% Witte's peptone) to which was added 1% of the various fermentable substances. The medium was tubed in Durham fermentation tubes, so the gas production could be observed as well as acid production. The mediums were sterilized by the fractional method. The length of the period of incubation was 5 days at a temperature of 37 C.

In selecting the medium, it was decided to use as simple a medium as possible without meat and without the use of a regulator (dibasic potassium phosphate). On the supposition that carbohydrate may be utilized first and then protein it was thought the results obtained in this medium would show best the results of carbohydrate metabolism. However, as will develop later, the results obtained did not altogether bear out this hypothesis.

Standards for determination of H-ion concentration were prepared as recommended by Clark and Lubs,¹ and the appropriate indicators covering the range P_H 8 to 4.4 used.

Referring to Table 3, it will be noted that the *Proteus vulgaris* cultures from the feces behave uniformly as regards fermentation reactions. Dextrose was the only substance fermented with acid and gas production in 5 days in the medium used. Other cultures showing the same fermentation reactions include 110, 111, 112 from water, 75, wound bandage, 76, dog's saliva, and laboratory cultures 1, 4, 64, 67, 70, 71, 92.

Another type of fermentation reactions includes those in which saccharose and maltose were fermented in addition to dextrose. In this group are included all of the *Proteus vulgaris* cultures from meat, 34, 78, 113, 114; one culture from water, 79; two cultures from necropsied animals, 53 and 94; 3 laboratory cultures, 2, 3 and 65. In this group may also be included stock culture 77, which showed a reading of P_H 5.6 in saccharose and 5.8 in maltose without production of gas in either. Gas production in dextrose consisted of only a bubble of gas, indicating that this strain was a feeble gas producer.

None of the typical *Proteus vulgaris* cultures fermented lactose, mannite or raffinose.

In general the cultures derived from meat and decomposing animal matter acted on carbohydrates more vigorously than strains from fecal sources. This is in accord with the observations of Cantu.

The behavior of the cultures classified as proteus species and *B. cloacae* in carbohydrate mediums was variable, and as a rule these cultures were more active in the breaking down of carbohydrate substances than *Proteus vulgaris*. Culture 51 from the peritoneal fluid of a guinea-pig and Culture 68 isolated from a child's stool were both very active as to gas production from carbohydrates. They correspond throughout in regard to acid and gas production with the exception of lactose, in which no gas was produced by Culture 51, while a small amount was produced by Culture 68—the P_H value being the same, 5.4 in both cases. These cultures were similar in other respects

TABLE 3
FERMENTATION REACTIONS

No. of Culture	Dextrose		Lactose		Saccharose		Maltose		Mannite		Raffinose	
	pH	Gas	pH	Gas	pH	Gas	pH	Gas	pH	Gas	pH	Gas
Proteus vulgaris												
Feces 11.....	4.9	+	7.6	—	7.4	—	7.3	—	7.4	—	7.6	—
17.....	5.0	+	7.7	—	7.6	—	7.4	—	7.4	—	7.6	—
29.....	5.1	+	7.8	—	7.6	—	7.5	—	7.4	—	7.6	—
55.....	4.8	+	7.5	—	7.6	—	7.4	—	7.5	—	7.6	—
96.....	5.5	+	7.6	—	7.3	—	7.4	—	7.6	—	7.4	—
98.....	5.3	+	7.5	—	7.6	—	7.6	—	7.6	—	7.5	—
99.....	5.0	+	7.4	—	7.5	—	7.5	—	7.5	—	7.4	—
103.....	5.0	+	7.6	—	7.3	—	7.3	—	7.5	—	7.5	—
108.....	5.0	+	7.3	—	7.5	—	7.4	—	7.4	—	7.6	—
115.....	5.0	+	7.4	—	7.4	—	7.6	—	7.7	—	7.6	—
Meat 34.....	5.1	+	7.8	—	5.3	+	5.2	+	7.5	—	7.5	—
78.....	5.3	+	7.6	—	5.3	+	5.3	+	7.5	—	7.5	—
113.....	5.1	+	7.4	—	5.3	+	5.2	+	7.7	—	7.7	—
114.....	5.3	+	7.5	—	5.1	+	5.2	+	7.8	—	7.6	—
Water 79.....	5.3	+	7.8	—	5.4	+	5.5	+	7.5	—	7.6	—
110.....	4.8	+	7.4	—	7.3	—	7.3	—	7.8	—	7.5	—
111.....	4.8	+	7.4	—	7.3	—	7.6	—	7.6	—	7.6	—
112.....	4.8	+	7.3	—	7.5	—	7.6	—	7.6	—	7.7	—
Necropsied animals												
53.....	5.3	+	7.8	—	5.3	+	5.7	+	7.5	—	7.6	—
94.....	5.2	+	7.6	—	5.3	+	5.1	+	7.6	—	7.6	—
Wound bandage 75..	5.1	+	7.7	—	7.5	—	7.5	—	7.5	—	7.5	—
Dog's saliva 76.....	4.9	+	7.6	—	7.5	—	7.5	—	7.5	—	7.6	—
Lab. cultures												
1.....	4.9	+	7.6	—	7.4	—	7.4	—	7.7	—	7.6	—
2.....	4.8	+	7.6	—	5.3	—	5.7	—	7.5	—	7.5	—
3.....	5.4	+	7.6	—	6.7	+	5.9	+	7.5	—	7.7	—
4.....	5.0	+	7.5	—	7.4	—	6.9	—	7.4	—	7.6	—
64.....	5.1	+	7.7	—	7.6	—	7.4	—	7.6	—	7.6	—
65.....	5.1	+	7.6	—	5.3	+	5.3	+	7.5	—	7.5	—
67.....	4.7	+	7.6	—	7.5	—	7.4	—	7.5	—	7.5	—
70.....	5.2	+	7.0	—	7.6	—	7.4	—	7.5	—	7.6	—
71.....	4.8	+	7.6	—	7.1	—	7.6	—	7.7	—	7.6	—
77.....	5.3	+	7.5	—	5.6	—	5.8	—	7.4	—	7.4	—
92.....	4.8	+	7.5	—	7.3	—	7.4	—	7.5	—	7.5	—
Proteus species												
Feces 20.....	4.8	+	7.5	—	7.6	—	7.6	—	7.7	—	7.8	—
Blood 69.....	5.1	+	7.8	—	7.6	—	5.3	+	5.4	+	7.6	—
Air 49.....	4.8	—	7.1	—	5.1	—	5.2	—	5.3	—	7.5	—
Meat 63.....	4.8	+	7.6	—	4.9	+	5.1	+	4.9	+	6.2	+
Lab. culture 80.....	5.4	—	7.2	—	5.7	+	6.4	+	7.3	—	7.5	—
B. cloacae												
Necropsied animal 51	5.3	+++	5.4	—	5.3	+++	5.1	+++	5.1	+++	5.0	+++
Feces 68.....	5.5	+++	5.4	+	5.0	+++	5.3	+++	5.1	+++	5.1	++
Meat 45.....	4.8	+	4.9	+	4.9	+	5.0	+	5.0	+	4.9	+
B. zopfii												
B. proteus zopfii 73..	7.3	—	7.4	—	7.1	—	7.4	—	7.5	—	7.5	—
Proteus zenkeri 85...	7.3	—	7.0	—	7.1	—	7.3	—	7.5	—	7.5	—
Pseudomonas protea												
Filtered water												
Ps. protea 86.....	5.3	++	7.7	—	7.7	—	5.3	+	5.3	+	7.7	—
87.....	5.0	++	7.7	—	7.7	—	5.2	+	5.4	+	7.7	—
Water (filtered ?) 24..	5.1	++	7.8	—	5.1	+	5.5	+	5.5	+	7.6	—

culturally and in agglutination tests and correspond to the type *B. cloacae*. They both showed a strong positive Voges-Proskauer reaction and produced no indol. Culture 45 should perhaps also be classed here. This organism is included in the study inasmuch as in the preliminary tests made just after isolation this organism failed to produce an appreciable amount of gas in lactose and corresponded in general to typical *Proteus vulgaris*. At the time H-ion tests were made, after the organism had been under cultivation a considerable length of time, gas production was quite pronounced in lactose broth. Two cultures from the same source showed this variation, though but one is included in this study.

The remaining cultures—20 (feces), 63 (meat), 69 (blood), 49 (air?) and 80 (laboratory culture)—are irregular in their fermentation reactions, and probably represent different species. Cultures 20 and 80 agreed with the typical fecal *Proteus vulgaris* cultures in fermentation of carbohydrates, but did not conform in all other respects. Culture 63 (meat) fermented all of the carbohydrates tested except lactose and Culture 69 (blood) fermented maltose and mannite but failed to ferment saccharose. Culture 49 produced no gas in any of the carbohydrates tested, but produced acid in dextrose, saccharose, maltose and mannite. This culture, however, produced gas in dextrose and saccharose broth in the preliminary tests. These cultures differed in other respects also from the typical *Proteus vulgaris* cultures.

DISCUSSION OF FERMENTATION REACTIONS

A survey of the fermentation reactions by *Proteus vulgaris* strains in the peptone carbohydrate mediums shows that dextrose is fermented by all; lactose, raffinose, and mannite by none, and saccharose and maltose are variable.

It should be emphasized that the results obtained in saccharose broth are not identical with those obtained in the peptone medium. It is probable that the meat extract used in broth mediums contains substances (hormones?) which increase the metabolic activities of certain strains as regards carbohydrate substances. This may apply also to maltose. Burton and Rettger in an investigation of the fermentation properties of high-ratio cultures of the aerogenes-cloacae type, found that when mediums containing Witte's peptone without meat extract was used the sugar utilization was never complete, but when Eimer and Amend peptone was used the sugar disappeared without the aid of meat extract. They refer to the variable amino-acid content of the two peptones as the reason for this difference. Witte's peptone was made use of in this study, and whether different results would have been obtained with other peptones was not determined.

As indicated by the preliminary tests all of the typical cultures produced a certain amount of gas in saccharose broth, though this was not always evident in the first two days, as in the case of dextrose, in most of the cultures. All, however, showed gas in 7 days. In the hydrogen-ion concentration tests in the peptone mediums only the cultures from decomposing meat and animal matter, one from water and several stock cultures produced acid and gas.

CORRELATION WITH RESULTS OBTAINED BY OTHER WORKERS

Berthelot, who has probably investigated most extensively the proteus group, also used a peptone solution as a basis for carbohydrate mediums. All of his strains (principally from diarrheic material) produced acid from dextrose, and none from lactose and mannite. Fermentation of saccharose was determined

at the end of 2, 5, 10 and 15 days, and while some strains showed a neutral reaction in 5 days, all were acid in 10-15 days. Maltose was variable in its behavior.

Horowitz, in a study of cultures isolated in an epidemic of gastro-enteritis, found glucose fermented by all strains with acid and gas production, maltose by all except one and mannite by all except one, though loss of power to ferment maltose was observed in some cultures after they had been kept under cultivation for some time. Saccharose was attacked by 7 out of 24 cultures. The author does not state whether the medium used contained meat extract or not. Three strains isolated from river water, which were agglutinated by a serum obtained by immunizing rabbits with one of the fecal cultures, failed to ferment maltose and mannite.

Larson and Bell found that pathogenic strains from various sources produced acid and gas in maltose.

Thjøtta studied a culture isolated from stools of a child ill with dysentery-like symptoms and found that the organism fermented glucose, saccharose, maltose and mannite at the time of isolation, though it lost its power of fermenting maltose and mannite after a short time.

Archibald isolated a strain from a case of choleraic diarrhea which fermented maltose and mannite, but not saccharose.

From the evidence previously given it seems probable that fecal strains often ferment maltose, saccharose and mannite when freshly isolated and later lose the power to a greater or less extent and sometimes completely.

In my study opportunity was not afforded for testing the cultures immediately on isolation, since a number of the strains were collected in the routine examination of feces for other purposes, and the study as a whole not attempted until a number of cultures had been obtained. It is thus possible that the cultural characteristics may have been altered considerably since the time of their isolation. According to the results obtained by Horowitz the power to ferment certain substances may be lost in as short a period as four weeks.

A much more extended investigation immediately on isolation of strains isolated from feces and from meat and water would be of value in throwing more light on the changes in metabolic activities as regards various carbohydrates.

Fermentation tests with various other carbohydrates are recorded in the literature. Glenn found that the monosaccharids, mannose and galactose, were fermented in addition to the carbohydrates previously referred to. Berthelot used galactose and levulose as test substances, and found that all cultures produced acid from galactose, but results were variable with levulose.

Archibald records acid and gas in glucose, mannite, levulose, maltose, galactose and dextrin, and no change in lactose, saccharose, dulcitol, adonite, inulin and raffinose by a strain derived from a case of choleraic diarrhea.

Horowitz records no action on dulcitol by the culture which he observed.

In my study several of the strains collected at the beginning of the work were tested in a large number of carbohydrate and related substances (Table 4). A broth medium neutral to litmus and containing 1% of the test substances and litmus and tubed in Durham fermentation tubes was used. The 7-day readings are recorded. It was found that the reduction of the litmus interfered considerably with the readings of acid production. Table 4 is included as being of a certain value, though the use of a number of the test substances was discontinued in a further study of the group. The results obtained indicate that levulose, galactose, glycerin and xylose, in addition to dextrose and saccharose, are fermented by all strains of *Proteus vulgaris*.

TABLE 4
RESULTS OBTAINED IN THE STUDY OF STRAINS TESTED IN A LARGE NUMBER OF CARBOHYDRATES
AND RELATED SUBSTANCES

	Monosaccharids				Disaccharids			Trisaccharids		Polysaccharids		Alcohols				Tetraoxyaldehyds			Hydroxybenzene	Gluco-sid	
	Levulose		Galactose		Saccharose	Lactose	Maltose	Raffinose	Dextrin	Inulin	Glycerin	Erythrite	Adonite	Dulcitate	Mannite	Sorbitol	xylose	Ara-binose	Isodulcitate	Inosite	
	Dextrose																				
Proteus vulgaris																					
Feces 11.....	+	r	+	r	+	a	+	-	-	-	+	-	-	-	-	-	+	-	-	-	-
17.....	+	r	+	r	+	a	+	-	-	-	+	-	-	-	-	-	+	-	-	-	-
29.....	+	r	+	r	+	a	+	+	-	-	+	-	-	-	-	-	+	-	-	+	-
Meat 34.....	+	r	+	r	+	a	+	+	-	-	+	-	-	-	-	-	+	-	-	-	-
Lab. cultures 1.....	+	r	+	r	+	a	+	+	-	-	+	-	-	-	-	-	+	-	-	-	-
2.....	+	r	+	r	+	a	+	+	-	-	+	-	-	-	-	-	+	-	-	-	-
3.....	+	r	+	r	+	a	+	+	-	-	+	-	-	-	-	-	+	-	-	-	-
4.....	+	r	+	r	+	a	+	+	-	-	+	-	-	-	-	-	+	-	-	-	-
Proteus species 20.....	+	a	+	a	+	a	+	-	-	-	-	-	-	-	-	-	+	-	-	-	-
47.....	+	r	+	r	+	a	+	-	-	-	-	-	-	-	+	+	-	-	-	-	-
B. cloacae 45.....	+	a	+	a	+	a	+	+	-	-	+	-	-	-	-	+	+	+	+	+	-
Ps. protea 24.....	+	a	+	a	+	a	+	+	-	-	+	-	-	-	+	+	-	-	-	-	-

+ = gas; - = no gas; a = acid; r = litmus reduced.

VOGES-PROSKAUER REACTION

The Voges-Proskauer reaction as a test for the identification of *Proteus vulgaris* is referred to by few authors. Archibald found that the organism isolated by him in a case of choleraic diarrhea gave a positive Voges-Proskauer reaction. This organism was typical in other respects, except that it produced a greenish fluorescence on agar, and rendered milk acid, and then alkaline, without curdling. The medium used was a dextrose peptone solution. He classifies the organism as *B. Proteus fluorescens*. This author refers to the statement of Orr that organisms of the proteus group frequently give a positive Voges-Proskauer reaction. Kligler,² in a study of 6 laboratory strains, found that all gave a negative Voges-Proskauer reaction.

In making the Voges-Proskauer test on my cultures, the same medium was made use of as in testing for hydrogen-ion concentration, namely 1% peptone solution containing 1% of dextrose. After an incubation period of 5 days at 37 C., tests were made by rendering the solution alkaline by the addition of 10% solution of potassium hydroxid and observations made at the end of 6 and 24 hours. None of the *Proteus vulgaris* cultures gave a positive test. Of the cultures classed as proteus species one culture, 49 (air) showed a positive test. Cultures 51 and 68 classed as *B. cloacae* gave decided positive tests. The *Pseudomonas protea* gave negative tests.

INDOL

Tests for the production of indol were made in accordance with the method recommended in the Standard Methods for Water Analysis of the American Public Health Association (1917). Tryptophan not being available, tests were made on various peptones to determine which gave the strongest tryptophan reaction by the bromin test. Armour's peptone was selected as being most favorable and the medium made up with 5% of this peptone, and 0.5% of dipotassium hydrogen phosphate in 1,000 cc of distilled water. After 2 days' incubation at 37 C., tests were made using paradimethyl-amino-benzaldehyde and concentrated hydrochloric acid.

Among *Proteus vulgaris* cultures the four isolated from meat (34, 78, 113, 114), one from water (79), the two from necropsied animals (53, 94), and laboratory cultures 3, 65 and 77 gave a positive indol test and the remaining were all negative. This correlates almost perfectly with the fermentation reactions in saccharose and maltose, the cultures recorded as producing a positive indol reaction consistently fermenting these carbohydrates. Culture 77 was recorded as giving a trace of indol, and this culture though producing no gas from saccharose or maltose peptone solution, showed a H-ion concentration represented by a P_H value of 5.6 and 5.8 in the respective sugars.

The cultures classified under proteus species except 63 and the *Pseudomonas protea* cultures gave positive tests for indol, while the *B. cloacae* and *B. zopfii* cultures were negative.

Cantu calls attention to the fact that the production of indol is more marked in cultures derived from animal sources which is borne out by the results obtained.

Horowitz comments on the fact that strains which gave a positive indol test as a rule fermented saccharose.

The results obtained by various workers in testing for indol are not in agreement. Feltz, who first made a careful study of the indol reaction, carried out tests with a number of different varieties of peptone and obtained very divergent results, some giving good positive reactions, some moderately good

and some negative with the same strains. He demonstrated that the presence of dextrose delayed the production of indol and also that acid interfered with the reaction, which was confirmed by Glenn. As a result of his tests, however, he considers the production of indol one of the distinguishing characteristics of *Proteus vulgaris*. In doubtful cases he recommends distillation, indol distilling very readily in the presence of watery vapor.

Herter and Ten Broeck found indol present in moderate amounts in the case of cultures derived from putrefying material. Indol-acetic acid was present in cultures 4 days old. Larson and Bell report indol present in perceptible proportions by strains from pathologic sources.

Kligler,¹ in studying the cultural characteristics of 5 laboratory strains of *Proteus vulgaris*, found that 3 produced indol (glycerin negative) and 2 produced no indol (glycerin positive).

Van Loghem and van Loghem-Pouw found that a strain of *Proteus* isolated from the urine of a diabetic patient was indol negative, and the same organism was later isolated by them from an intestinal abscess. These two strains produced a reddish violet color on testing for indol by the nitrite test, but this color was shown to be different from the color of nitrosoindol, by spectrum analysis. Other indol tests with these organisms were negative. These investigators further isolated 3 strains among 30 *proteus* strains derived from intestinal contents and feces, which showed the same reaction. A new species, *B. proteus anindologenes*, is proposed by these authors to cover this group of organisms. Absence of interagglutination of the two types is cited as added evidence of basic differences.

Berthelot, who made a detailed and accurate study of the indol reaction of strains derived principally from cases of infantile diarrhea, demonstrated that the non-indol producing strains acting in a tryptophan medium produced at least indol-3-acetic acid, and on this basis a separation should not be made into the two species *Proteus vulgaris* and *B. proteus anindologenes*.

Horowitz found that 7 out of 24 strains gave a strongly positive reaction for indol in 24 hours, and the remaining 17 did not give a positive reaction even in 6-7 days. The medium used and method of testing for indol are not described.

Thjøtta tested a strain of *Proteus vulgaris* at the time of isolation and found it to be indol positive, but a month and a half later it failed to produce indol in either broth or peptone water, at the same time exhibiting loss of power of fermenting maltose and mannite.

In my study no tests were made for the presence of indol-acetic acid, nor was distillation resorted to in order to determine indol. It is possible that some of the strains tested immediately on isolation would have shown a positive indol test. The correlation between the indol positive and the maltose and saccharose positive organisms is so striking that it seems certain that the two reactions are closely related in cultures of *Proteus vulgaris*.

REDUCTION OF NITRATES

Tests were made on the cultures studied for reduction of nitrates, by the methods recommended in the Standard Methods of Water Analysis of the American Public Health Association (1912), the medium consisting of 0.1% peptone medium containing 0.02% nitrite free potassium nitrate. The incubation period was for 4 days at 37 C. Tests were made with sulphanilic acid solution and α -amidonaphthelene acetate solution. Tests for ammonia were made with the same medium by adding a few drops of Nessler's solution. All

of the cultures tested with the exception of the cultures of *Proteus zenkeri* gave positive tests for nitrites and ammonia.

UREA DECOMPOSITION

Tests were not made on the cultures studied for decomposition of urea, but for the sake of completeness, reference is made to the work of Brodmeier, who records the active decomposition of urea by *Proteus vulgaris* in neutral and alkaline solutions, and to Horowitz, who found that fecal strains transformed urea energetically with the evolution of ammonia in large amounts.

HYDROGEN SULPHID

The production of hydrogen sulphid by *Proteus vulgaris* cultures in peptone mediums containing lead acetate has been recorded by Horowitz. This was previously demonstrated by several other authors.

AGGLUTINATION OF PROTEUS CULTURES BY PROTEUS SERUM

There is little agreement among various workers in regard to the matter of agglutination of *Proteus vulgaris* by immune serum, some authors finding that only the strain used for immunizing is agglutinated, while others have been able to show that an immune serum produced by one strain may also influence other strains. Klieneberger¹ has reviewed the literature on this subject.

Rodella tested an immune serum produced from a strain isolated by Silberschmidt in a food poisoning epidemic against seven other strains. The strains used are not described except for the statement that all belonged to the variety *Proteus vulgaris*, and one was isolated from a wound and another from the stool of a child ill with enteritis. The latter culture was the only one which gave a positive result, and that occurred in as high a dilution as the homologous organism.

Grossmann found that an organism isolated in a case of cystitis was agglutinated by an immune serum produced from a strain isolated in a case of peritonitis in a dilution of 1:750, while the immune serum from the cystitis organism agglutinated the other in 1:400. Immune serums produced by *Proteus vulgaris* isolated from meat agglutinated the pathogenic strain from cystitis in a dilution of only 1:20.

Weber studied the agglutination of three strains derived from decaying meat and found interagglutination in dilutions of 1:10 to 1:50, but no agglutination except with the homologous organism in dilutions above 1:50.

Klieneberger¹ studied the agglutination reactions of a number of strains from different sources, including several from cystitis urine, one from brain abscess, two from meat and two which he classified as *mirabilis* from urine and meningitis pus. The immune serums from four strains from pathologic processes agglutinated the strain used for immunizing and also other strains from pathologic sources. As a result of his work this author considers proteus strains as forming a biological entity in the matter of agglutination just as *B. typhosus* and *B. paratyphosus*. He found that as a rule strains derived from meat were not influenced by serums derived from pathogenic sources, but that serums derived from meat strains did influence pathogenic strains. He considers this behavior as indicative of differences between saprophytic and parasitic forms and of gradual adaptation of saprophytic forms to the animal body.

Glaser and Hachla tested the agglutination reactions of a number of strains derived from sausage and meat, and also several laboratory strains including *Proteus vulgaris*, *Proteus mirabilis* Kral, *Proteus zenkeri* and *B. piscicidus versicolor*. These authors concluded from the results obtained that the action was individualistic like that of *B. coli*.

Cantu tested a number of strains derived from various sources, including normal and diarrheic human stools, spoiled meat, vegetables, etc., and succeeded in obtaining agglutination with the homologous organism in a dilution of only 1:10, while other strains were not at all agglutinated except in one instance.

Horowitz studied the agglutination reactions of a number of strains derived from diarrheic stools and separated them into five groups by the method of testing all the strains with a serum derived from one strain, then testing the remaining strains with a serum derived from one of the strains not agglutinated by the first serum and so on, until a serum had been found for all the different strains.

Agglutination tests in my work were carried out with immune serums obtained by inoculating rabbits with suspensions of several different cultures. Cultures representing different habitats were selected. These included No. 29 (feces), 34 (putrefying meat), 75 (wound bandage), 4 (laboratory strain), and 27 *Ps. protea* (water).

Inoculations for the production of immune serums were carried out as follows:

First inoculation: 1 cc of suspension in salt solution of killed 24-hour culture subcutaneously.

Second inoculation, 5 days later: 1 to 2 cc of suspension of live culture of organism subcutaneously.

Third inoculation, 5 days later: 2 cc of suspension of live culture of organism subcutaneously.

Fourth inoculation, 5 days later: 2 cc of suspension of live culture of organism intraperitoneally.

Bled 10-12 days after the last injection.

A serum of titer 1:20,000 from Culture 29 was obtained, 1:1,000 from 34, 1:10,000 from 75, 1:2,000 from 27 and 1:30,000 from 4.

In performing the agglutination tests a 2-hour incubation period at 37 C. was used. The tubes were then kept at a temperature of 15 C. overnight and the readings made 18-24 hours after the test was begun.

The suspensions of organisms used in testing the serums for agglutinins were prepared as follows: 24-hour cultures grown on agar slants were washed off with 0.85% salt solution containing 0.1% of formalin. The suspensions were kept at cold room temperature for 3 days with daily shakings. The suspensions were then diluted by the addition of salt solution containing formalin as above to contain about $1\frac{1}{2}$ billion organisms per cc in the final test, the standardization being made by comparison with a typhoid-paratyphoid vaccine this number of organisms.

Readings were made as follows:

++++ = Organisms all precipitated, supernatant fluid perfectly clear.

+++ = Organisms nearly all precipitated, supernatant fluid slightly cloudy.

++ = Organisms partially precipitated, supernatant fluid more cloudy than preceding.

+

— = No precipitate or clearing (like control without serum).

TABLE 5
AGGLUTINATION TESTS OF CULTURES WITH *PROTEUS VULGARIS* IMMUNE SERUMS

	Serum 4 (Titer 1:30,000)	Serum 29 (Titer 1:20,000)	Serum 75 (Titer 1:10,000)	Serum 34 (Titer 1:1,000)	Serum 27 (<i>Ps. protea</i>) (Titer 1:2,000)
<i>Proteus vulgaris</i>					
Feces 11.....	1:2,000	1:2,000	1:5,000	1:500	0
17.....	1:10,000	1:2,000	1:1,000	1:100	0
29.....	1:200	1:20,000	1:500	0	0
55.....	1:500	1:20,000	1:5,000	0	0
96.....	1:5,000	1:10,000	1:2,000	1:1,000	0
98.....	1:100	1:10,000	1:2,000	no test	no test
99.....	0	1:5,000	1:500	1:1,000	0
103.....	1:5,000	1:20,000	1:2,000	1:200	0
108.....	1:100	1:2,000	1:500	1:200	0
115.....	1:100	1:5,000	1:1,000	1:500	no test
Meat 34.....	1:30,000	1:20	1:10,000	1:1,000	0
78.....	0	0	0	0	0
113.....	0	0	0	0	no test
114.....	0	1:2,000	1:500	1:200	0
Water 79.....	1:5,000	1:5,000	1:200	1:1,000	0
110.....	1:100	1:10,000	1:500	0	0
111.....	1:200	1:10,000	1:500	0	0
112.....	1:500	1:500	1:200	0	0
Necropsied animals 53.....	1:5,000	0	1:200	no test	0
94.....	1:20,000	0	1:5,000	1:1,000	0
Wound bandage 75.....	1:10,000	0	1:10,000	1:1,000	0
Dog's saliva 76.....	1:5,000	0	1:5,000	1:1,000	0
Laboratory cultures 1.....	1:500	0	1:5,000	1:50	1:50
2.....	1:30,000	0	1:10,000	1:500	0
3.....	1:1,000	1:1,000	1:2,000	1:200	0
4.....	1:30,000	0	1:5,000	1:500	0
64.....	1:1,000	1:200	1:5,000	1:500	0
65.....	1:5,000	0	1:10,000	1:1,000	0
67.....	1:200	1:1,000	1:5,000	0	0
70.....	1:5,000	0	1:2,000	1:200	0
71.....	1:5,000	0	1:2,000	1:200	0
77.....	1:500	1:1,000	1:1,000	1:1,000	1:20
92.....	1:10,000	1:20,000	1:2,000	1:200	no test
<i>Proteus species</i>					
Feces 20.....	1:200	0	0	0	0
Blood 69.....	0	0	0	0	1:40
Air 49.....	0	0	0	0	0
Meat 63.....	0	0	0	0	0
Laboratory culture 80.....	0	0	0	0	0
<i>B. cloacae</i>					
Necropsied animals 51.....	0	0	0	0	0
Feces 68.....	0	0	0	0	0
Meat 43.....	0	0	0	0	0
<i>B. zopfi</i>					
<i>B. proteus zopfi</i> 73.....	1:100	0	0	0	0
<i>Proteus zenkeri</i> 85.....	0	0	0	0	0
<i>Ps. protea</i>					
Filtered water <i>Ps. protea</i> 86...	0	0	0	0	1:400
87...	Spontan.	Spontan.	Spontan.	Spontan.	1:100
Water (filtered ?) 24.....	1:200	1:40	0	0	1:2,000

In Table 5 the highest dilution in which agglutination occurred with the various organisms is recorded. These readings correspond to +, in case the next higher reading was ++, or to ++ if the next lower was —.

Preliminary tests of all cultures were made with the various serums in a dilution of 1:20, and if a positive result was obtained tests were made in higher dilutions: 1:50, 1:100, 1:200, 1:500, 1:1,000, 1:2,000, 1:5,000, etc., the highest dilution used depending on the titer of the serum.

A review of the results obtained with the 5 different immune serums and the various cultures as antigens brings out the following points:

Serums 4 (laboratory culture) and 75 (wound bandage) in general agglutinate the same cultures, there being only a few exceptions. Serum 34 (meat) which had a comparatively low titer (34, 1:1,000; 4, 1:30,000; 75, 1:20,000) agglutinated most of the cultures agglutinated by Serums 4 and 75, but in correspondingly lower dilutions. In many cases cultures were agglutinated by this serum in exactly 1/10th the dilution in which the same cultures were agglutinated by Serums 4 and 75 (cf. 11, 17, 3, 4, 64).

Cultures 4, 34 and 75 interagglutinated with the respective serums to the titer limit, except that 4 was agglutinated by Serum 34 in a dilution 1:500 instead of 1:1,000.

Serum 29 (feces) did not agglutinate 4, 34 or 75, but its homologous organism was agglutinated by Serums 4 and 75 in low dilutions (4, 1:200; 75, 1:500). Apparently the agglutinins of Serum 29 produced no receptors for 4, 34 or 75, but the agglutinins of Serums 4 and 75 did produce receptors for 29. It is to be expected, therefore, that strains agglutinated by Serum 29 should also be agglutinated by Serums 4, 34 and 75, and such proves to be the case with a few exceptions.

The agglutination of all the fecal cultures by Serum 29 is striking. This serum also agglutinated Culture 114 derived from meat, 79, 110, 111, 112 from water, and laboratory cultures 3, 64, 67, 77, and 92, though several of these were agglutinated in comparatively low dilutions (112, 64). Of the fecal cultures, Nos. 96, 98, 99, 103, 108, 115 were of recent isolation at the time of testing, while Cultures 11, 17, 29, 55 had been carried on artificial mediums for a considerable time. Apparently the length of time of artificial cultivation was not a factor in agglutination, since agglutination in equally high dilutions was obtained with old and with freshly isolated cultures.

All of the *Proteus vulgaris* cultures were agglutinated by Serum 75 in dilutions varying from 1:200 to 1:10,000, with the exception of 78 and 113 (cultures derived from meat). Most of these cultures were also agglutinated by Serum 4, though results were not always consistent, in some cases Serum 75 agglutinating in higher dilutions than 4 and vice versa.

The general deduction may be drawn from the results obtained with the cultures studied that two or more types may be established on the basis of agglutination reactions. Cultures 29, 110 and others may be cited as examples of one type, that in which agglutination was obtained in high dilutions with a serum produced by a culture derived from feces. Cultures 34, 94, 4 and others are examples of another type which is not agglutinated by such a serum. On this basis Cultures 78 and 113, which were not agglutinated by any serum, belong to still other types.

Whether this division into types is justifiable cannot, however, be determined without further evidence. Horowitz subdivided a number of cultures derived from feces, river water and meat into 5 different groups on the basis of agglutination reactions, all of which 5 groups contained cultures of fecal

origin. This author suggests variation in agglutinability of cultures kept under laboratory conditions.

The results obtained, while not exhibiting as clear cut a division into types as might be desired, nevertheless demonstrate conclusively that agglutination by an immune serum derived from one culture is not limited to the homologous culture. Variable degrees of adaptation to the animal body as suggested by Klieneberger¹ offers a possible explanation of the diversity of results obtained. Variability may also have a bearing on the matter. Cultures from different sources which show practically the same agglutinations with the 5 serums used may be selected from Table 5 (34 from meat and 94 from necropsied animal), (29 from feces and 11 from water), (75 from wound bandage and 76 from dog's saliva), but on the other hand, there are numerous discrepancies as regards agglutination with the various serums used among cultures derived from the same source.

CORRELATION OF FERMENTATION AND AGGLUTINATION TESTS

A comparison of the results obtained in the fermentation tests and the agglutination tests shows that correlation is present to a limited extent. Cultures derived from feces which were alike in fermentation reactions were agglutinated somewhat similarly. The cultures derived from meat, however, which had the same fermentation reactions throughout, showed three different sets of agglutination reactions, Culture 34 agglutinated in high dilutions by Serums 4, 34 and 75, but not by 29; Cultures 78 and 113 agglutinated by none of the serums used, and Culture 114 agglutinated in the highest dilution by Serum 29.

Of the cultures isolated from water, 79 corresponded to the cultures isolated from meat in fermentation reactions and 110, 111 and 112 corresponded to the fecal types. The three latter cultures were like the fecal type in agglutination reactions, and 79 was also probably more closely related to this type than the other.

Cultures 53 and 94 (necropsied animals) agreed with the cultures derived from meat in fermentation reactions, while 75 (wound bandage) and 76 (dog's saliva) were like the fecal type, but all showed similar agglutination reactions.

The above results confirm those obtained by Horowitz, who found similar variations among cultures derived for the most part from feces. The immune serums from strains which failed to ferment saccharose and maltose and gave a negative indol reaction agglutinated strains which fermented saccharose and maltose and gave a positive indol reaction, and strains which were alike in fermentation reactions exhibited different agglutination properties. Apparently strains may be closely related and yet differ in their fermentation reactions or agglutination properties. It is problematical as to what extent agglutination and fermentation reactions are correlated in this as in other groups of organisms. Hefferan found that a high degree of interaction as regards agglutination properties existed in a group of organisms more or less like *B. prodigiosus* which showed the same fermentation reactions.

The results obtained in the case of the organisms grouped under proteus species, *B. cloacae*, *B. zopfii* and *Ps. protea* when tested with immune serums derived from the strains of *Proteus vulgaris* used, are conspicuous by being negative throughout with few exceptions. (Immune Serum 4 agglutinated Culture 20 derived from feces in a dilution of 1:200 and *B. proteus zopfii* in 1:100.) These results correlate with cultural differences between these groups and *Proteus vulgaris*. The serum derived from *Ps. protea* 27 agglutinated very few of the *Proteus vulgaris* cultures, and these in low dilutions (Culture 1

in 1:50, 77 in 1:20, and 69 in 1:40). *Ps. proteus* 86 was agglutinated in a dilution of 1:400, and Culture 24 derived from the same source as 27 in a dilution of 1:2,000.

In addition to the above experimental work on the agglutination reactions of immune serums with the strains used in this study, further consideration of agglutination behavior of *Proteus vulgaris* as recorded in the literature is presented in the following pages.

AGGLUTINATION OF *PROTEUS VULGARIS* BY THE SERUM OF PATIENTS INFECTED WITH *PROTEUS* STRAINS

Agglutination of the organism concerned in cases of suppurative infections, cystitis, pyelonephritis and others are recorded in the literature. Klieneberger¹ and Frost have reviewed the works of Pfaundler, who demonstrated positive agglutination of *Proteus vulgaris* in intestinal catarrh, Wolf in cystitis and suppurative process of gonorrheal origin, Grassberger in suppurative infection, Lubowski and Steinberg in mastoid infection, Jochmann in suppurative mastoid infection, Haim in typhoid fever, Doernig in a case of food poisoning.

The more recent literature includes the work of Klieneberger,² who found cultures of *Proteus vulgaris* isolated in cases of urinary infection agglutinated by the serum of the patients in dilutions of 1:80 and 1:320. In a case of cystopyelitis the infecting strain was agglutinated by the serum of the patient in dilutions of 1:1,280 and 1:2,560. The high agglutination he considered probably due to general infection. The same author reports a case of sinus thrombosis due to *Proteus vulgaris* in which the organism was agglutinated in a dilution of 1:320.

Flinzer describes a case of rib abscess in which *Proteus vulgaris* was isolated, and in which the serum of the patient agglutinated the organism in a dilution of 1:6000.

Maymone cites the case of a general septicemia following a gunshot wound in which *Proteus vulgaris* was isolated from the blood and which was agglutinated by the serum of the patient in a dilution of 1:2,500. A case of proteus meningitis and proteus sepsis in a new-born child is described by Goebel, in which *Proteus vulgaris* in pure culture was isolated from the blood and spinal fluid and which was agglutinated in a dilution of 1:60.

Thjotta reports a case of pyemia with abscesses of the supra- and infra-clavicularis in which *Proteus vulgaris* isolated from the pus was agglutinated in a dilution of 1:80. By the complement fixation test an amount as low as 0.0063 of the patient's serum produced strong fixation, while a normal serum required 0.1.

Agglutination of the serum of patients with intestinal infections with proteus strains are described by several authors, though others have obtained negative results in such cases.

Archibald isolated an organism in a case of choleraic diarrhea classified by him as *B. proteus fluorescens* which was agglutinated by the patient's serum in low dilutions. Mandel describes an organism in a food poisoning epidemic which he identifies as *Proteus vulgaris*, but concerning the classification of which there seems to be some question, which was agglutinated by the serum of patients in a dilution of 1:25.

Bertrand, who studied organisms of the proteus group isolated from cases of infantile diarrhea, was unsuccessful in demonstrating clearly agglutination of the organisms by the blood of infants from whom the organisms were isolated. He was only able to obtain and then only irregularly an agglutination as high as 1:20.

Horowitz in a study of *Proteus vulgaris* concerned in an epidemic simulating dysentery, was unable to show agglutination of the organism even in a dilution of 1:25, in explanation of which fact he thinks the presence of the organism in the intestine may have been of such short duration, that there was not sufficient time for the elaboration of specific antibodies.

Thjøtta isolated a culture of *Proteus vulgaris* in a case of diarrhea resembling dysentery which was not agglutinated in a dilution even as low as 1:10 by the serum of another patient suffering with pyemia due to proteus infection.

AGGLUTINATION OF PROTEUS STRAINS BY THE SERUM OF PATIENTS SUFFERING FROM WEIL'S DISEASE

Jaeger has ascribed to an organism classified by him as *Bacillus proteus fluorescens* resembling *Proteus vulgaris* except in the matter of greenish pigment, an etiologic rôle in Weil's disease. This organism was isolated from the urine, blood and organs of patients dead of the disease. Altho not now generally accepted as the causal agent, agglutination of *Proteus fluorescens* and *Proteus vulgaris* by the serum of patients has been reported by several authors. The works of Lüdke and Pfaundler in this connection are referred to by Frost, both of these authors finding the serum of patients agglutinating the organism in dilutions of 1:20 or higher. Brüning isolated an organism which he describes as *Bacillus proteus fluorescens* from the urine and feces of a patient with infectious icterus (Weil's disease) in which the organism was scarcely agglutinated in a dilution of 1:50. The serum of the patient, however, agglutinated *B. typhosus* strongly in a dilution of 1:50. Abeles tested human icterus serum and did not obtain agglutination in dilutions higher than 1:40.

AGGLUTINATION OF PROTEUS CULTURES BY THE SERUM OF TYPHOID FEVER PATIENTS

A review of the literature dealing with agglutination of proteus cultures by the serum of patients with typhoid fever as well as the agglutination of the typhoid bacillus by the serum of patients with proteus infection has been included by Frost in his work on the agglutination of *Ps. protea* by the serum of typhoid fever patients.

Abeles also investigated the action of human typhoid serum on 6 proteus strains derived from enteritis stools. Seventeen cases were studied, and in only 2 cases was an agglutination as high as 1:40 obtained, while in the others agglutination rarely exceeded 1:10.

In my work a number of tests were made on specimens of serums obtained from the various Marine hospitals in this country. These were tested for agglutination reactions against *B. typhosus* Rawling, and those serums which gave positive results were then tested against a number of strains of *Proteus vulgaris* and a strain of *Ps. protea*. Several of the strains not agglutinated by *B. typhosus* and therefore considered as normal in this respect, were also tested against *Proteus vulgaris* cultures.

The cultures used in the first set of tests included the laboratory strains 1, 2, 3, 4, and Culture 11, isolated from feces. These were all tested against the same serums. Of these, Cultures 3 and 11 showed no agglutination with the serums used, except that 11 was agglutinated in a dilution of 1:10 by one of the serums. Cultures 1, 2, 4 showed agglutination in varying degrees, as indicated by Table 6.

Agglutinations were recorded at the end of 1 hour and in 24 hours, a 1-hour incubation period being used. The tubes were then placed at cold room tem-

TABLE 6
AGGLUTINATION TESTS OF TYPHOID-AGGLUTINATING SERUMS WITH *PROTEUS VULGARIS*
AND *PSEUDOMONAS PROTEA*

Serum	B. typhosus (Rawling)						Culture 86 (P.s. protea 362t)						Culture 1 (Proteus vulgaris I)						Culture 2 (Proteus vulgaris II)						Culture 4 (Proteus mirabilis)					
	1:10		1:20		1:40		1:10		1:20		1:40		1:10		1:20		1:40		1:10		1:20		1:40		1:10		1:20		1:40	
	1 hr.	24 hr.	1 hr.	24 hr.	1 hr.	24 hr.	1 hr.	24 hr.	1 hr.	24 hr.	1 hr.	24 hr.	1 hr.	24 hr.	1 hr.	24 hr.	1 hr.	24 hr.	1 hr.	24 hr.	1 hr.	24 hr.	1 hr.	24 hr.	1 hr.	24 hr.	1 hr.	24 hr.	1 hr.	24 hr.
7051	4+	4+	4+	4+	4+	4+	2+	3+	+	2+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
7052	3+	4+	4+	4+	4+	4+	2+	3+	+	2+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
7053	—	4+	4+	4+	4+	4+	2+	4+	+	3+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
7086*	4+	4+	4+	4+	4+	4+	2+	4+	+	3+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
7103	+	3+	3+	3+	3+	3+	2+	4+	+	2+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
7106†	2+	4+	4+	4+	4+	4+	2+	4+	+	2+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
7107	2+	4+	4+	4+	4+	4+	2+	4+	+	2+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
7112	2+	3+	3+	3+	3+	3+	2+	4+	+	3+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
7118	3+	4+	4+	4+	4+	4+	2+	4+	+	3+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Control	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
No serum	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—

1:80 1:160 1:320
* Patient received one inoculation of typhoid vaccine one week before blood was drawn.
† 7106 + 2+ — 2+ — +

perature over night. Tests in this series were made only in dilutions of 1:10, 1:20, and 1:40. The record of agglutination in the highest dilution, 1:40, furnished a comparison of the relative agglutinating powers of the various sera.

The serums which agglutinated *B. typhosus* strongly, 7051, 7086, 7106 and 7118, agglutinated *Ps. protea* somewhat less strongly (Table 6). *Proteus vulgaris* 1 was agglutinated by these serums also less strongly than *B. typhosus*, corresponding more closely to *Ps. protea*, with the difference that practically no agglutination of the *Proteus vulgaris* culture was evident in 1 hour, though in 24 hours the reaction was marked. *Proteus vulgaris* Cultures 2 and 4 were not agglutinated by any of these serums except by 7118, the reactions being less marked than with Culture 1.

TABLE 7

SHOWING RESULTS OBTAINED WITH TYPHOID AGGLUTINATING SERUMS AND STRAINS OF *PROTEUS VULGARIS* AND *PSEUDOMONAS PROTEA*

	Serum 7245 (1:160)						Serum 7254 (1:320)					
	1:10		1:20		1:40		1:10		1:20		1:40	
	2 hr.	24 hr.	2 hr.	24 hr.	2 hr.	24 hr.	2 hr.	24 hr.	2 hr.	24 hr.	2 hr.	24 hr.
<i>Proteus vulgaris</i>												
Feces 11.....	—	—	—	—	—	—	—	—	—	—	—	—
17.....	—	—	—	—	—	—	—	—	—	—	—	—
29.....
55.....
Meat 34.....
35.....
Laboratory cultures 1*	—	++	—	++	—	++	—	+	—	+	—	+
2	—	—	—	—	—	—	—	—	—	—	—	—
3	—	—	—	—	—	—	—	—	—	—	—	—
4	—	—	—	—	—	—	—	—	—	—	—	—
64
65
67
<i>Proteus species</i>												
Feces 20.....	—	—	—	—	—	—	—	—	—	—	—	—
Air 49.....
Meat 63.....
<i>B. cloacea</i>												
Feces 68.....
<i>Ps. protea</i>												
Water 24...	+	++++	—	++++	—	+

1:80 1:160

* Laboratory culture 1: — ++ — +

The remaining serums, 7052, 7053, 7103, 7107 and 7112, agglutinated *B. typhosus* in lower dilutions than the preceding serums. The results obtained with the *Ps. protea* culture and these serums were as high as those with *B. typhosus*, and in the case of Serum 7112 higher. The results with *Proteus vulgaris* 1 corresponded in general with those obtained with the *B. typhosus* and *Ps. protea* cultures, but as before no agglutination was evident in 1 hour. Cultures 2 and 4 were agglutinated by Serum 7052, but by none of the others.

A larger number of cultures were then tested with two serums which agglutinated *B. typhosus* in comparatively high dilutions (Serum 7245 in 1:160 and 7254 in 1:320). None of the cultures tested except *Proteus vulgaris* 1 and No. 24 (*Ps. protea*) gave positive results (Table 7). The results obtained with the latter culture confirm the results obtained by Frost.

AGGLUTINATION OF *PROTEUS VULGARIS* CULTURES BY NORMAL HUMAN SERUM

Agglutination of *Proteus vulgaris* by normal serums has been considered by several authors. Lannelongue and Achard found in only one case tested by them strong agglutination of *Proteus vulgaris* by normal serum. Klieneberger¹ tested 6 normal serums against 22 strains of *Proteus* and found none positive except some strains of *B. zopfii* and *Proteus zenkeri*. Abeles tested serums of patients with bronchitis, tuberculosis, gastro-enteritis, and paralysis, classing these under normal human serums, against 6 *proteus* strains and found none agglutinated in higher dilutions than 1:40.

In my study serums which were known to be normal as regards typhoid agglutination, namely, showing negative agglutination with *B. typhosus*, were tested with a culture of *Ps. protea* and Cultures 1, 2, 3, 4, and 11 of *Proteus vulgaris* (Table 8). Positive agglutinations in dilutions of 1:20 to 1:40 (with one exception, Serum 749) were obtained with the culture of *Ps. protea* and *Proteus vulgaris* 1, though as before agglutination of the *proteus* culture was not evident before 24 hours. A comparison with the results obtained with the serums agglutinating *B. typhosus* shows that agglutination took place in higher dilutions with the typhoid serums than with the normal serums. *Proteus* cultures 2, 4 and 11 were not agglutinated by any of the serums except with 7050 (dilution 1:20). This corresponds in general with the behavior of these cultures with typhoid serums.

The results of the tests made indicate that occasional cultures of *Proteus vulgaris* may be agglutinated by the serum of typhoid fever patients, but agglutination takes place, in general, in lower dilutions and is not as characteristic as with the homologous organism or even as with *Ps. protea*. It is not apparent why certain other cultures of *Proteus vulgaris* which are alike as regards cultural behavior and agglutination by *Proteus* immune serums do not behave similarly.

The same culture (*Proteus vulgaris* 1) which was agglutinated by typhoid serums also showed a tendency to be agglutinated by serums which were normal as regards typhoid agglutination, positive results being obtained in somewhat lower dilutions.

AGGLUTINATION OF TYPHUS SERUM WITH *PROTEUS VULGARIS*, THE SO-CALLED WEIL-FELIX REACTION

The agglutination of certain strains of *Proteus vulgaris* by the serum of patients suffering from typhus exanthematicus has recently been described by Weil and Felix. These organisms were isolated from the urine of patients suffering from the disease. Agglutination occurs regularly in dilutions of 1:100 to 1:2,000. These strains are described as differing from the saprophytic strains of *Proteus vulgaris*, though such strains may be agglutinated by a serum artificially produced by the strains in question. Epstein and Morawetz reported agglutination in dilutions as high as 1:10,000 occasionally. Dietrich tested the serum of 81 typhus fever patients and 100 normal persons and found that in the former class the serums of all agglutinated the organism in dilutions of 1:100 to 1:6,400, while in the latter class all failed to agglutinate in dilutions up to 1:50 and 1:100. Paneth found the agglutinins present in the serum of typhus fever patients to be transitory.

The high dilutions in which agglutination occurs in typhus fever cases is noteworthy, being higher than in cases reported of infection with *Proteus vulgaris* itself or in cases of typhoid fever or in Weil's disease.

TABLE 8
AGGLUTINATING TESTS OF NON-TYPHOID-AGGLUTININATING SERA WITH *PROTEUS VULGARIS*
AND *PSEUDOMONAS PROTEA*

Serum	B. typhosus (Rawling)	Culture 86 (Ps. protea 362t)	Culture 1 (Proteus vulgaris I)	Culture 2 (Proteus vulgaris II)	Culture 4 (Proteus mirabilis)
	1:10 1:20 1:40 1 hr. 1 hr. 1 hr.	1:10 1:20 1:40 1 hr. 1 hr. 1 hr.	1:10 1:20 1:40 1 hr. 1 hr. 1 hr.	1:10 1:20 1:40 1 hr. 1 hr. 1 hr.	1:10 1:20 1:40 1 hr. 1 hr. 1 hr.
7039	-	+?	- +? -	- +? -	- +? -
7050	-	- 2+ -	- 2+ -	- 2+ -	- 2+ -
7054	-	- 2+ -	- 2+ -	- 2+ -	- 2+ -
7088	-	- 3+ -	- 3+ -	- 3+ -	- 3+ -
7090	-	- 3+ -	- 3+ -	- 3+ -	- 3+ -
7114	-	- 2+ -	- 2+ -	- 2+ -	- 2+ -
7119	-	- 4+ -	- 4+ -	- 4+ -	- 4+ -
7120	-	- 2+ -	- 2+ -	- 2+ -	- 2+ -
7122	-	- 4+ -	- 4+ -	- 4+ -	- 4+ -
No serum	-	-	-	-	-

PROTEIN METABOLISM

Putrefaction and the proteolytic enzyme of *Proteus vulgaris* have been the object of study of a number of investigators, including Feltz, Herter and Ten Broeck; Glenn; Drummond; Kendall and Walker; Sperry and Rettger; Rettger, Berman and Sturges; Rettger and Newell; Tissier and Martelly; Berthelot, and others.

It has been common to consider *Proteus vulgaris* among the true putrefactive bacteria, or those which decompose native albumin. Rettger, Berman and Sturges, however, have shown that the decomposition of complex proteins like egg albumin, as well as of albumoses and peptones by certain organisms with proteolytic and putrefactive properties including *Proteus vulgaris*, is due primarily to the proteolytic enzyme elaborated by such organisms, and not to the direct action of the bacteria themselves. Mediums containing purified egg albumin and dialyzed proteoses were not attacked if the inoculations were made with very few organisms or from cultures less than 24 hours old in which there had not been sufficient time for the elaboration of the proteolytic enzyme.

Drummond states that peptone is necessary in the medium for the elaboration of the enzyme, though the results obtained by Rettger and associates indicate that the amino acids contained in peptone are utilized for this purpose. If these are available for furnishing the nitrogen necessary for bacterial development, the enzyme elaborated may bring about complete hydrolysis of native proteins.

Feltz demonstrated that the presence of glucose in mediums inhibited the production of indol, an index of proteolytic action. Glenn has shown that the presence of acid in mediums produced as the result of the breaking down of the carbohydrate was the factor concerned. Liquefaction of gelatin likewise was inhibited by the presence of carbohydrates including dextrose, saccharose, levulose, mannose, galactose, maltose and raffinose. Rettger, Berman and Sturges also showed that the presence of 1% of glucose in peptone mediums inoculated with gelatin-liquefying bacteria prevented reduction in the amounts of proteoses and peptones for a period of two weeks.

Kendall and Walker studied the action of the bacteria-free enzyme, obtained by filtration of plain broth or gelatin cultures through unglazed porcelain, on dextrose gelatin and found that the liquefaction of gelatin by the proteolytic enzyme was not inhibited by the presence of the dextrose, and that moderate amounts of organic acids also did not interfere.

Tissier and Martelly and Glenn describe the enzyme as tryptic. Glenn considers the enzyme of *B. cloacae*, on the other hand, to be peptic in its action.

Herter and Ten Broeck demonstrated the fact that the curdling of milk was due to the enzyme elaborated by the organism and not to acid production. By neutralizing the acid formed with CaCO_3 , they found that coagulation still took place. Kendall, Day and Walker showed that the action of proteus on milk proteins was very vigorous, about 6% of the total nitrogen content being decomposed in 3 weeks.

OCCURRENCE

Proteus vulgaris is usually considered to be very widespread in its distribution, but whether it is ubiquitous to the extent that certain authors consider it, has not been definitely established. Decomposing organic matter, particularly of animal origin, appears to be the habitat by preference of this organism. Its occurrence in water, in the soil, in the digestive tract is probably not as frequent as is commonly supposed.

Burton and Rettger in a study of coli-like organisms isolated from 1,000 samples of soil, leaves, twigs, flowers, bark of trees, berries, snow, etc., comment on the fact that no recognizable members of the proteus group were encountered, and conclude that their habitat is elsewhere than in the soil. These authors used the method of inoculating all samples into glucose broth tubes, which should favor the development of *Proteus vulgaris*.

Rogers, Clark and Evans in a study of 166 coli-like organisms from grains, including corn, barley, wheat and oats, include a group of 7 cultures, of which 6 appear to correspond to the *Proteus vulgaris* type. These authors also made use of glucose broth in the isolation of their organisms. The results obtained indicate that *Proteus vulgaris* is relatively much less frequent than the *B. coli* or *Lactis aerogenes* types of organisms on grains.

A number of authors have observed the relatively infrequent occurrence of *Proteus vulgaris* in normal feces. This matter has been considered at greater length in this paper under the heading Pathological Occurrence.

Meat.—The presence of *Proteus vulgaris* in partially decomposed meat has been observed on numerous occasions. Cantu tested 22 specimens of such meat and found *Proteus vulgaris* present in all. A number of food poisoning epidemics have been ascribed to the presence of *Proteus vulgaris* in meat products. These are considered under a separate heading. The occurrence of *Proteus vulgaris* in sausage and chopped meat which had not yet reached the stage of noticeable decomposition has been noted by a number of authors. Cantu found the organism present in 33.3% of samples of raw sausages. Sacquépée and Loygue found bacteria belonging to the proteus group present in 36% of samples of sausages and other meat products, *B. paratyphosus* being present only once, and Cary found *Proteus vulgaris* present in 33% of sausages tested. Zweifel reports the occurrence of 23 organisms which resembled paratyphoid B, but which he states in reality belonged to the proteus group in 248 samples of raw chopped meat tested.

Water.—Jordan in a study of 543 organisms isolated from river water, includes 23 which correspond to *Proteus vulgaris* type. Cantu tested 80 samples of potable water and 1.25% gave positive results for the organism.

Digestive Tract of Animals.—Berthelot reports *Proteus vulgaris* always present in the feces of normal rats. Choukévitch showed it to be present in the intestinal contents of horses in 25% of tests made. The organisms were present, however, in relatively small numbers, and it was necessary to use a considerable amount of material which was allowed to putrefy to permit of the development of putrefactive organisms. Cantu found *Proteus vulgaris* present in the excrement of 4% of hens whose feeding was normal and in 66.6% of those which were fed with meat. Jordan² reports the occurrence of proteus vulgaris in swine feces in the proportion of 41 out of 725 colonies isolated from 58 hogs. Most of these were isolated by a preliminary growth in brilliant green broth, followed by plating on Endo medium.

Soil.—Though Burton and Rettger consider the occurrence of *Proteus vulgaris* rare in soil, Cantu reports 44.2% samples of soil positive for the organism. The presence of decaying organic matter of animal origin in soil has a bearing on the subject.

Miscellaneous Food Substances.—Cantu investigated the presence of *Proteus vulgaris* in a number of food substances and found that the organism occurred on celery, melons, bananas, in cheese and other foods. Two hundred samples of milk were tested and 3.5% contained the organism.

Mouth Secretions, etc.—*Proteus vulgaris* has been shown to be present in the mouth secretions by Feltz, and Cantu. The latter author also found the organism present in a small percentage of tests of the skin.

OCCURRENCE, PATHOLOGICAL

The occurrence of *Proteus vulgaris* in pathologic conditions has been very completely covered in the review of the literature of the proteus group up to the year 1898 by Meyerhof. This includes the occurrence of the organism in mixed infections, infection with the organism alone, local and general. Mixed infections with streptococci are described in suppurative phlegmons, parametritis, puerperal fever and meningitis; with staphylococci in parametritis, osteomyelitis, meningitis, brain abscess; with pneumococci in pneumonia and gangrene of the lungs; with *B. coli* in cystitis. Pure infection is described in proteus cystitis, pyelonephritis, abscesses, pleuritis and peritonitis. Among general infections are included infections due to meat poisoning, as well as Weil's disease, though in the latter case, the etiology has recently been established on another basis.

In the more recent literature a number of infections both primary and secondary are described. The following is a partial résumé of such infections.

Eye Infections.—These are described by Hanke and Tertsch, and Wirtz, the case described by the former being an infection with an unusually virulent strain of *Proteus vulgaris*, and the latter a case of infection with *B. tetani* and several other organisms including *Proteus vulgaris* as secondary agents. Larson and Bell¹ in a study of *Proteus vulgaris* derived from pathologic sources include one from an eye infection following cataract operation.

Ear Infections.—Klieneberger² describes an infection by *Proteus vulgaris* in sinusthrombosis in which the serum of the patient agglutinated the organism in a dilution of 1:320. Lauffs found *Proteus vulgaris* present 6 times out of 26 in mastoid abscesses and their complications, 2 times in pure culture and once with streptococci and diplococci. Urbantschitsch isolated *Proteus vulgaris* in a perisinus abscess.

Other Suppurative Processes.—*Proteus vulgaris* in brain abscess is described by Leutert, in sublingual abscess by Ware, in rib abscess by Flinzer, in pustule simulating anthrax by T. Orr, in periurethral abscesses by Bertelsmann and Mau, in uterine abscess by Broughton-Alcock, in a laparotomy wound by Larson and Bell.¹ Ungermann found *Proteus vulgaris* in 37% of 38 diseased appendices, but also in 2 normal, and always with other organisms, and therefore considered it only of secondary importance. The presence of *Proteus vulgaris* in purulent war wounds in association with other organisms has been described by Doyen and Yamanouchi, Tissier, Goadby and Stewart.

Infections of the urinary tract are described by Klieneberger,² Jeffreys, van Loghem, and Saathof. The latter author considers mixed infection of *B. coli* and *Proteus vulgaris* as more severe than of *B. coli* alone. Geraghty finds organisms of the proteus group usually associated with alkaline cystitis.

Osteo-periostitis of the inferior maxillary caused by *Proteus vulgaris* is recorded by Domínguez.

Grossmann describes a case of general peritonitis, in which *Proteus vulgaris* of high virulence was isolated.

A case of gas gangrene in which the bacillus of malignant edema was associated with *Proteus vulgaris* is described by Heyde. Larson and Bell² refer to a culture of *Proteus vulgaris* isolated in a case of gas gangrene of the lungs.

The presence of *Proteus vulgaris* in the circulating blood has been recorded by several authors. Jochmann isolated *Proteus vulgaris* and streptococci from the blood of a patient with a suppurative mastoid infection. Bertelsmann and Mau demonstrated the presence of *Proteus vulgaris* in large numbers in the blood in a patient suffering from periurethral abscess. Libman and Celler in a study of a number of cases of otitis media found the organism once in blood cultures. Maymone describes a case of septicemia due to proteus infection following a gunshot wound. Pauly describes a wound infection with *Proteus vulgaris*. Following an operation pleural infection was evident and the organism in pure culture was isolated from the resulting empyema. This author considers it possible, in the light of the above case and from a review of the literature, that a local tissue necrosis in a patient whose condition otherwise is bad may increase the virulence of *Proteus vulgaris* and allow its passage into the blood. Larson and Bell describe *Proteus vulgaris* isolated from the heart's blood of a patient who had died from peritonitis following a gunshot wound of the intestines.

OCCURRENCE, PATHOLOGICAL — DIGESTIVE TRACT

The occurrence of *Proteus vulgaris* in the digestive tract is a matter which has received a considerable amount of attention. For the sake of convenience, in this discussion the occurrence of the organism in the normal digestive tract will be considered together with its pathologic occurrence, although strictly it should not perhaps be here included.

Certain authors state that *Proteus vulgaris* is a habitual inhabitant of the intestinal tract just as *B. coli*. This statement, however, is not borne out by the investigations of several recent workers. It seems certain that numerically *Proteus vulgaris* is much less abundant than *B. coli* in normal feces. In my work this organism was isolated only 5 times in 80 tests made by streaking pooled specimens of feces from several subjects, directly on Endo plates, Endo medium having been shown to be a favorable medium for the growth of *Proteus vulgaris*; *B. coli*, on the other hand, was present in practically all specimens.

Cantu found *Proteus vulgaris* in 30% of normal human stools. Feltz as a result of his researches decided that *Proteus vulgaris* in feces occurs only rarely. In 12 healthy subjects he isolated it in only one case. He ascribes the rare occurrence of the organism in feces to the fact that the gastric juice plays an antiseptic rôle with organisms introduced by means of food.

In an investigation of several thousand samples of feces from dysentery convalescents, Stewart found *Proteus vulgaris* present less than a dozen times, and considers proteus uncommon as an inhabitant of the intestinal tract.

A number of authors have considered the occurrence of *Proteus vulgaris* in the stools of normal infants. Bertrand studied 24 specimens from healthy infants and found the organism twice. Metchnikoff considers normal infants in whose stools the organism is present as carriers of *Proteus vulgaris*. He reports two such "carriers" among 6 normal infants. In an institution 18, or 57%, of 33 children proved to be "carriers." This percentage has been considerably lower than among infants suffering from gastro-enteritis, of which 96% were found by this author to have the organisms in their stools. Bahr examined the feces of 27 normal children and found *Proteus vulgaris* in two cases. Horowitz examined the dejecta of 40 infants and was unable to isolate the organism a single time.

The occurrence of *Proteus vulgaris* in diarrheic stools, on the other hand, has been frequently noted, especially in infants. Cantu found it in 40% of diarrheic stools. Horowitz, who investigated an epidemic which was of the

nature of dysentery, being characterized by bloody stools, demonstrated *Proteus vulgaris* in 24 out of 63 cases, or 38%. Dysentery, pseudo-dysentery or typhoid organisms could not be isolated from the stools and the serum of patients failed to agglutinate the dysentery bacillus. As a result of his studies this author believes *Proteus vulgaris* penetrating into the intestinal tract, either with contaminated water or food, may provoke gastro-enteritis.

Infantile Diarrhea.—*Proteus vulgaris* as concerned in infantile diarrhea in this country has been described only occasionally. Booker as a result of his investigations of the subject decided that not a single organism was responsible, but that streptococci and *Proteus vulgaris* were of most frequent occurrence. On the other hand, a number of European workers report the frequent occurrence of *Proteus vulgaris* in diarrheic stools of infants.

Bertrand studied the stools of 55 infants suffering from infantile diarrhea (in London) and was able to demonstrate *Proteus vulgaris* in all. Metchnikoff studied 217 cases of infantile diarrhea covering a period of four years and isolated the organism in 204, or 96%. He attributes the infection of infants to carriers and flies. Gildemeister and Baerthlein studied infantile diarrhea in Berlin and isolated *Proteus vulgaris* in 31% of 70 cases. Tsiklinsky investigated infantile diarrhea in Moscow and Paris, including 70 cases in the former locality and 8 in the latter, during a period of four years. *Proteus vulgaris* was isolated in 65% of the cases studied, sometimes in almost pure culture. This author also isolated it in 20% of normal feces, but found these cultures not virulent for rabbits, in comparison with the cultures isolated in the diarrheic cases, $\frac{1}{4}$ - $\frac{1}{2}$ c.c. of which invariably killed rabbits and guinea-pigs when inoculated subcutaneously. Thjøtta isolated a culture of *Proteus vulgaris* from the stools of a child with dysentery symptoms.

The matter of symbiosis of *Proteus vulgaris* with other organisms in infantile diarrhea has been considered by several authors. Berthelot studied the association of *Proteus vulgaris* with *B. aminophilus intestinalis*, an organism belonging to the *lactis aerogenes* group, and found that either organism alone produced no ill effect in rats on an exclusive milk diet, but when administered together, diarrheic symptoms were produced in 6-8 days. Metchnikoff found that nursing rabbits receiving by mouth mixed cultures of the two organisms, *Proteus vulgaris* and *B. welchii*, developed symptoms similar to experimental cholera. Tsiklinsky isolated *B. perfringens* in 10% of 78 cases (*Proteus vulgaris* in 65%) and considers symbiosis of these organisms plays an important rôle in infantile diarrhea.

PROTEUS VULGARIS IN FOOD POISONING EPIDEMICS

The relation of proteus to food poisoning epidemics has been discussed by various authors. Epidemics ascribed to this organism have been of very much less frequent occurrence than similar epidemics ascribed to members of the paratyphoid group.

Food poisoning epidemics, in which this organism has been considered to bear a causal relationship, described in the literature are recorded in Table 9. In all of these epidemics with two exceptions meat products have been concerned. Dieudonné reports an epidemic in which infected potato salad was incriminated, and Ohlmacher attributes one to the eating of infected oatmeal.

In certain of these epidemics the organism was isolated from the food product and an effort was made to establish its causative relation by feeding experiments with the culture as well as by feeding the suspected food. In several other epidemics the organism was isolated from the stools of patients, and tests made on animals with the culture thus obtained.

In the epidemic described by Levy, pure cultures of the organism were obtained on gelatin plates planted with vomitus and stools of a patient who later died. Animals injected died of hemorrhagic diarrhea. The same organism was isolated from slime in the bottom of an ice chest in which the meat in question was considered to have become infected. The blood of animals tested was sterile and the author considers the effects produced to have been due to the production of the toxic substance, sepsin, in the decomposed meat rather than to infection.

Wescenberg isolated an organism which corresponded to Hauser's *Proteus vulgaris*, except that he states it was more virulent for animals and failed to produce indol. He believes the causal relationship of the organism to the epidemic was established by the fact that the organism was found so exclusively in the meat tested, and that broth cultures in 0.2 cc amounts injected subcutaneously killed mice in the same way as the infected meat. However, no tests were made on the vomitus and stools of the patients.

Ohlmacher describes an epidemic which he ascribes to the eating of oatmeal which he believed had been infected with *Proteus vulgaris*. The oatmeal prepared the evening before and warmed the following morning had been probably contaminated with dust from falling plaster. None of the suspected food or the vomitus of patients was available for examination, but the organism was isolated from the plaster dust.

Silberschmidt sought to establish the relationship of the organism in the epidemic investigated by him by bacteriologic examination of the sausage in question and of samples of "good" sausages as controls, and by feeding and inoculation experiments with the meat and with the organism isolated. *Proteus vulgaris* was not isolated a single time from the control sausages plated directly on gelatin, and only once from such sausages planted first in broth and then on gelatin. The organism was present in large numbers in the suspected sausages. The suspected sausages in the dry state fed to mice and cats produced no ill effects, but if incubated in broth for several days and fed in the moist state caused death of mice and guinea-pigs in 2 days. Broth cultures of the organism in the suspected sausages injected subcutaneously caused the death of white mice in 7-24 hours, while injection of control cultures from the "good" sausages sometimes, but not always, caused death. Guinea-pigs and rabbits died in 5-9 days when injected subcutaneously with the cultures from the suspected sausages. *Proteus vulgaris* was recovered in several cases from the peritoneal fluid and from the heart blood of the injected animals. The author also attempted to determine the presence of toxic substances in the meat by extraction, and injecting animals with the extract, but obtained inconclusive results. He considers the harmful effects due to the multiplication of the organism in the intestines, with the formation of toxic substances; in other words, he believes that infection is accompanied by intoxication, but that infection is of primary importance.

Glücksman reports cases of food poisoning supposedly caused by the ingestion of smoked meat which had come from a sick hog, one patient succumbing. The organs from the body were received for bacteriologic examination, but were in such a state of decomposition that nothing definite could be established from their examination. Portions of the meat were also examined, and from these *Proteus vulgaris* was isolated, almost in pure culture. Mice injected with broth cultures in amounts varying from 0.1-1 cc died in 18 hours to 4 days, and showed symptoms of diarrhea, while guinea-pigs receiving (subcutaneously) 0.5-2 cc died in 7 days. Postmortem examination

Author	Place	Date	No. of Persons Affected	Organism	Suspected Source of Infection	Symptoms	Convalescence or Deaths	Animal Tests
Johns.....	Chemnitz, Germany	1886	About 160	Proteus mirabilis	Sausages.....	Aching of limbs, vomiting, malaise, diarrhea, headache, fever, dizziness	No effect on animals
Levy, E.	Strassburg, Germany	1893	18	Proteus	Meat kept in ice box which was unclean	Bloody vomitus and stools, great weakness, slight fever	1 death; others convalescent 2-4 weeks	Animals (inoculated?) died with symptoms of diarrhea
Wesenberg, G. ...	Mansfeld, Germany	1897	63	Chopped meat from sick cow. Eaten raw or not well cooked. Meat had been kept in damp, musty cellar	Diarrhea, headache, pain in limbs, muscular weakness, dizziness, faintness	1 death (child); others convalescent 5-7 days	Mice and guinea-pigs inoculated with the suspected in 18 hours to 3 days; 0.2 c c broth culture killed than 0.2 c c caused weakness
Ohlmacher, A. P.	Gallipolis, Ohio	1897	218	Proteus vulgaris	Oatmeal, which was cooked, allowed to stand over night and reheated. Contaminated by dust	Chills, aching of limbs, severe headache, nausea and vomiting, pain in abdomen, profuse diarrhea, dizziness, pulse 100-120, temperature 102.5-105	Convalescence in 4 days to 2 weeks	Suspected food not available. Oatmeal was artificially tainted with cultures of organism and treated by sterilization method. Extract caused septic peritonitis and 3 guinea-pigs (1/2-1 c c injected intraperitoneally)
Silberschmidt, W.	K. & St. G., Switzerland	1898	43	Proteus vulgaris	Sausages.....	Aching of limbs, chills, fever, vomiting, diarrhea, headache, thirst	1 death (18 yrs. old), some convalescent in 2-5 days, others 7-30 days	Feeding: Suspected sausages did not affect mice or guinea-pigs. Feeding suspected sausages in moist state after 12 days in broth caused death of white mice and guinea-pigs. Inoculation: Cultures of suspected sausages caused death of mice and guinea-pigs, rabbits, by subcutaneous, intraperitoneal and intravenous inoculations. Cultures of recovered organism injected subcutaneously caused death of mice, also killed guinea-pigs intraperitoneally. Extract of sausages was toxic for rabbits, especially injected intravenously. Broth cultures of organism caused death of mice with symptoms of diarrhea in 0.1 to 0.5 c c amounts injected taneously; 0.5 to 2 c c of broth cultures caused death of guinea-pigs in 6-7 days when injected subcutaneously. Feeding: Meat fed to mice caused death. Inoculation: Rabbit injected with 4 c c broth culture following day. Broth cultures heated to 65 C. injected in 0.5 c c amounts produced no effect. Filtrate of live cultures caused only temporary effect. Filtrate of young live cultures had slightly toxic effect. Rats fed with filtrate caused death of animals. Feeding: Rats and mice fed with sausage injected subcutaneously into animals (0.1 to 0.5 c c) caused death of mice. Feeding: Mice fed with potato salad died in 24 hours. Mice fed with artificially infected potatoes inoculated at 20-37 C. died in 24-48 hours. Broth cultures of organism fed to mice on bread had no effect. Inoculation: Mice and guinea-pigs injected subcutaneously with suspensions of agar cultures were slightly recovered. Subcutaneous injection of filtrate of culture of mice and guinea-pigs had no effect. Feeding experiments not conclusive
Glücksmann, S. ...	St. Gallen, Switzerland	1899	2	Proteus vulgaris	Smoke d, uncooked meat of sick hog	Acute gastro-enteritis (fever, vomiting, diarrhea) collapse, rapid pulse, palpable abdomen	1 death; 1 convalescent after 8 days	
Pfuhl, A.	Germany	1900	81	"Proteus mirabilis" (but fermented lactose more vigorously than dextrose (B. cloacae?))	Sausages.....	Vomiting, diarrhea, loss of appetite, weakness	Convalescent in 12 hours	
Schumburg.....	Hanover, Germany	1901	34	Proteus	Sausages.....	Malaise, diarrhea, weakness, vomiting	Convalescent in 12 hours	
Dieudonné, A.	Hannenburg, Germany	1903	150-180	Proteus vulgaris	Potato salad.....	Headache, dizziness, weakness, vomiting, collapse, aching of limbs, colic-like pain, pulse 88-92	Majority convalescent in a few hours, a few weak for several days	
Mayer, Mandel, H.	Germany	1912	46	Proteus vulgaris	Fish kept in unclean box	Chills, headache, aching of limbs, weakness, loss of appetite, vomiting, diarrhea, temp. 38-39.3 C., pulse 120, in one case herpes facialis, in three cases trace of albumin in urine	Early recovery	

showed enlarged spleen, congested intestines and adrenals. The organism was recovered in 2 animals out of 9. The author considered the effects produced as due to infection with the organism and intoxication by its metabolic products.

Pfuhl attributes an epidemic of food poisoning to the eating of infected sausages, in which a slowly liquefying organism producing swarm colonies in gelatin and which he classifies as *Proteus mirabilis* was isolated. He states, however, that the organism isolated did not correspond in all respects to Hauser's description of *Proteus mirabilis*. It produced gas in dextrose and lactose, but more actively in lactose than dextrose. No digestion of casein is described in milk. The organism appears to have been more closely related to *B. cloacae* than to *Proteus vulgaris*. In this case also the causal relationship was considered to have been established by the isolation of the organism from the suspected meat and by feeding and inoculation experiments, though the organism was not isolated from the stools of the patients. The author considered the harmful effects due rather to a toxin than to infection.

Schumberg, who investigated an epidemic involving 34 persons and due to eating infected sausages, isolated the organism from the suspected food. The sausage fed to rats and mice caused death in 24 hours with congestion of the intestines and enlargement of the spleen and liver. Only a few organisms were found in the blood, and the author considers the effects produced were due to toxic substances. The author examined several other kinds of sausages and was not able to isolate *Proteus vulgaris*.

Dieudonné recovered *Proteus vulgaris* from potato salad, which had caused an epidemic affecting 150-180 persons. In this case mice fed with the salad died in 24 hours, and *Proteus vulgaris* was isolated from the spleen and kidneys, while mice inoculated remained well. The author artificially contaminated potatoes with broth cultures of the organism isolated and allowed them to stand at various temperatures for 12 hours and then fed them to animals. Animals fed with potatoes which had been incubated at temperatures above 18 C. died. Meat was infected in the same way and similar results were obtained. Bread artificially contaminated in the same way had no effect on animals. The author therefore concludes that the organism itself was not pathogenic for animals, but that toxic substances were produced in the potatoes and meat which caused death.

Mayer described an epidemic due to the eating of spoiled fish. In this case stools of the patients were examined and *Proteus vulgaris* was isolated, which was agglutinated by the serums of the patients in a dilution of 1:25. This author also considers the harmful effects produced to have been due to toxic substances produced in the meat by the multiplication of the organism.

Mandel has reported the same epidemic and isolated an organism from the stools of patients which he states was undoubtedly *Proteus vulgaris*. The cultural characteristics of a number of the strains described appear to be somewhat variable. No gas production in dextrose, no curdling of milk and no liquefaction is recorded of some of the cultures, but laboratory strains of *Proteus vulgaris* used as controls show the same discrepancies and it is difficult to judge as to the significance of the results obtained.

In both accounts of the above epidemic the appearance of *Proteus vulgaris* in the stools was followed by that of members of the paratyphoid-enteritidis group. Mandel agrees with Mayer in considering the bacteria themselves much less harmful than the toxic substances produced by them in the food. The short incubation period and quick recovery point to an intoxication rather than to an infection.

A definite proof of the causal relationship of the organisms isolated to the illness is lacking in all of the above epidemics. The isolation of identical organisms from the suspected food and from the stools and vomitus of patients has not been reported in any case. The real cause of the effects produced in alleged cases of food poisoning by *Proteus vulgaris* has not been satisfactorily explained, some of the investigators attributing it to a multiplication of the organism itself in the body, and others to the toxic substances produced by it, either before or after entering the intestine. Levy, Pfuhl, Schumburg, Dieudonné, Mayer and Mandel incline to the view that toxic substances are produced in the food before its entrance into the body, and that these cause the injurious effects. Wesenberg considers multiplication of the organism in the body the cause of harm. Silberschmidt thinks the multiplication of the organism in the body and the formation of toxic substances in the intestine following this multiplication are both factors to be considered though infection is the more important. Glückmann considers that these injurious effects are due to infection and intoxication by the metabolic products which have been elaborated in the food substance before it enters the body.

The assumption that injurious effects are produced by the presence of toxic substances in the food, before it enters the intestine, is open to the criticism that partially decomposed meat in which it is probable that *Proteus vulgaris* is often present, since meat seems to be one of the natural habitats of *Proteus vulgaris*, has often been shown to produce no harmful effects. Levy considers that toxic substances are produced at only certain stages of decomposition. The short duration of illness and quick recovery in most cases, however, would seem to point to an intoxication either by substances produced before or after the food substance reaches the intestine, and most investigators incline to this view. Dieudonné found organisms in the blood, spleen and liver of animals fed with potatoes and meat artificially contaminated with broth cultures of *Proteus vulgaris* and kept 12 hours at temperatures above 18 C., but the organisms were few in number, and he considers infection as playing a minor rôle. Schumburg also found very few organisms in the blood and spleen of animals fed with meat artificially infected and incubated for 24 hours before feeding.

The presence of the organism in large numbers in the digestive tract after feeding has been demonstrated on the other hand by several authors, including Silberschmidt and Metchnikoff. The latter performed feeding experiments on chimpanzees and nursing rabbits. *Proteus vulgaris* was absent in the normal feces of chimpanzees, but chimpanzees fed with diarrheic material showed large numbers of the organism in the feces. The contents of the jejunum, ileus and cecum of one animal which died showed abundant cultures of the organism at necropsy. The same results were obtained with young rabbits. Chimpanzees and rabbits fed with pure cultures of *Proteus vulgaris* isolated in cases of infantile diarrhea died and large numbers of the bacilli were present in all parts of the digestive tract of the chimpanzee. Cultures of the organism administered with *B. welchii* provoked infections similar to cholera. Negative results as regards harmful effects produced by feeding with broth cultures have been reported by Archibald, Feltz, Herter and Ten Broeck, Meyerhof and Bahr. Further evidence is needed to establish the etiological rôle of *Proteus vulgaris* in cases of food poisoning.

Toxin.—Levy isolated organisms from putrefying beer yeast which he identified as *Proteus vulgaris*. By treating a liquefied gelatin culture of the organism with absolute alcohol, and precipitating with calcium chlorid and

drying, the toxic substance "sepsin" was obtained. This produced the same effect as living cultures of the bacteria, causing the death of dogs injected intravenously with 1 gm. of the sterile powder and of rabbits and guinea-pigs injected intravenously, intraperitoneally or subcutaneously with 0.2-0.3 gm. Symptoms of vomiting, bloody diarrhea and rise of temperature were present, and hemorrhagic infiltration of the intestines and enlargement of the spleen were present on necropsy.

Fornet and Heubner also investigated putrefying beer yeast and isolated an organism differing from *Proteus vulgaris* which produced sepsin and killed dogs, which they designated as *Bact. sepsinogenes*. By artificially contaminating putrefying beer yeast with cultures of *Proteus vulgaris* isolated from meat and human feces, these authors were not able to produce the symptoms in dogs described by Levy.

Meyerhof in studying the effects of cultures of *Proteus vulgaris* and of the filtrates of cultures on rabbits, mice and guinea-pigs, came to the conclusion that infection was concerned in the effects produced, since the organism was present in the blood and organs of the animals, but also that a sort of toxemia was produced as in tetanus, diphtheria, and botulism, the minimal lethal dose for mice corresponding to 0.1 of live culture, 0.5 of killed culture and 2 cc of filtrate.

Herter and Ten Broeck precipitated cultures of *Proteus vulgaris* with alcohol, centrifugalized and dried in vacuo over H_2SO_4 , dissolved in sterile salt solution and inoculated intraperitoneally into guinea-pigs and found the lethal dose to be 8.2-11.5 mg. per 100 gm. body weight. The toxin of *B. coli* prepared in the same way, however, was stronger, the minimal lethal dose being 4 mg. per 100 gm. body weight. The authors were not able to establish an immunity to the toxin.

Berthelot made an extensive series of experiments to ascertain the toxic properties of cultures of *Proteus vulgaris* isolated in cases of infantile diarrhea, and also of a culture isolated from putrefying material. Seven-day cultures grown in different kinds of mediums and sterilized by means of ether were tested on 500 gm. guinea-pigs, injected intravenously and intraperitoneally and the fatal dose determined. In general, the amount of culture required to produce death in 6-18 hours varied from 1-5 cc, and the culture from putrefying material as far as tested was not much less toxic than the one from the case of infantile diarrhea. The filtrate from cultures was tested in the same way and the fatal dose varied from $2\frac{1}{2}$ cc to more than 12 cc. The toxic action of dried bacterial cells was also tested. The results expressed in milligrams of bacterial cells before drying varied from 10-50 mg. injected intravenously producing death in 15-20 hours, and 25-300 mg. injected intraperitoneally producing death in less than 24 hours.

The results obtained by these writers indicate that *Proteus vulgaris* produces a very weak soluble toxin as compared with tetanus or diphtheria. It is more comparable with that produced by *B. paratyphosus*, recently shown by Ecker to be toxic to the extent that amount of 1 to 5 cc of filtrate of broth cultures injected intravenously caused the death of young rabbits.

PATHOGENICITY

The pathogenicity of *Proteus vulgaris* has been discussed by a number of workers. Though the organism is apparently often saprophytic in its nature, it has been shown also on numerous occasions to be parasitic. Stewart, who isolated *Proteus vulgaris* from septic wounds in 24% of cases studied, states

that "as a pathogenic agent this organism is now well recognized." The enumeration of pathologic processes by Meyerhof and by Klieneberger¹ in which *Proteus vulgaris* is concerned as a factor as well as a number of more recent reports of proteus infections referred to in this paper attest to the pathogenic properties of the organism under certain conditions.

A number of authors have differentiated between virulent and nonvirulent strains of *Proteus vulgaris*. Tsiklinsky states that cultures isolated in infantile diarrhea exhibited a high degree of virulence, killing guinea-pigs and rabbits in 24-36 hours when $\frac{1}{4}$ - $\frac{1}{2}$ c c was injected subcutaneously, while proteus isolated from normal feces had no effect on rabbits. Larson and Bell² found that freshly isolated cultures of proteus from human lesions were pathogenic for rabbits, rats and guinea-pigs, producing abscesses or granulomatous types of lesions, but that after being kept under laboratory conditions, such cultures produced no lesions. Large doses of these cultures, however, showed toxic properties when injected into laboratory animals. Cultures of *Proteus vulgaris* from decaying protein matter were not found to be pathogenic by these authors. They conclude that strains of *Proteus vulgaris* pathogenic for rabbits, rats and guinea-pigs are also pathogenic for man. Nonpathogenic strains could be rendered pathogenic by inoculation into the anterior chamber of the eye of a rabbit. Pauly, as referred to above, considers that a local tissue necrosis may increase the virulence of proteus.

Numerous tests on animals for pathogenicity are recorded in the literature by other workers. In general, the results obtained indicate that strains freshly isolated from pathologic sources, or such strains which have lost their virulence and been subjected to animal passage for increase of virulence may produce definite lesions including abscesses, enlargement of spleen, hemorrhage of the intestine and a diarrheic condition. Strains which are not pathogenic in the sense of producing lesions as noted above may in large doses produce toxic symptoms.

In my work, virulence tests were carried out by injecting mice with 24-hour broth cultures in 1 c c and 0.1 c c amounts to determine whether strains from certain sources were more virulent for mice than strains from other sources. In general, 1 c c of broth culture injected subcutaneously caused death of mice within 24 hours, and 0.1 c c produced no ill effects, regardless of source. The following cultures of *Proteus vulgaris* killed in 1 c c amounts but not in 0.1 c c: 17, 29, 55, 96 (feces); 78 (meat); 79 (water); 75 (wound bandage); 76 (dog's saliva); 2, 3, 4, 64, 67, 70, 71, 92 (laboratory cultures). Culture 108 from feces caused death in a 0.1 c c amount. The following cultures did not cause death in 1 c c amounts: 11 (feces); 34 (meat); 53 and 94 (necropsied animals); 65, 77, 80 (laboratory cultures). The cultures classified as *B. cloacae* 51, 68 and 45; and *B. zopfi* 73 and *Proteus zenkeri* 85 had no effect on animals in 1 c c amounts. Cultures 47 (air?), 63 (meat) and 69 (blood) classified as proteus species killed in 1 c c amounts but not in 0.1 c c. The cultures classified as *Ps. protea*, 86, 87 and 24 also were virulent in 1 c c amounts but not in 0.1 c c.

As far as investigated no definite lesions were present, and it is probable that the effects produced were due to toxicity of the cultures. Death of several rabbits injected with killed cultures for the production of immune serums ensued within 24 hours after injection, probably due to toxic action. Abscesses were present at the site of inoculation.

The results obtained by various workers who have tested cultures of *Proteus vulgaris* isolated in cases of food poisoning or from cases of infantile diarrhea, indicate that such cultures were not markedly pathogenic in com-

parison with the cultures used in this study. Glücksmann who caused death of mice in 18 hours to 3 days by the injection of 0.1-0.5 c c of broth cultures of an organism isolated in a food poisoning epidemic considers the organism to have been very pathogenic for animals. Levy, Schunburg and Silberschmidt used 0.1-0.5 c c of cultures to produce death. Wesenberg states that mice were killed with 0.2 c c of a 24-hour broth culture, and that less than 0.2 c c caused weakness and loss of appetite.

On the other hand, cultures of *Proteus vulgaris* isolated from certain other pathologic processes and tested immediately on isolation sometimes exhibit a high degree of virulence. Grossmann reports a culture isolated from a case of peritonitis which killed mice in amounts of 0.005 c c.

CLASSIFICATION

The criteria to be used in the classification of organisms in the proteus group do not seem to be well established in some cases. Kruse, whose classification consists of a number of groups, includes among these the proteus group, members of which are characterized as aerobes or facultative anaerobes of medium size decolorized or irregularly stained by Gram's stain. Spores are absent and colonies have a tendency to spread and occur with raylike extensions or with stellate outgrowths away from the colony, sometimes with the separation of daughter colonies. Decomposition of protein substances is accompanied by putrefactive odor. Morphologically, the organisms occur as varying from pleomorphic coccoid forms to long filaments. A supplementary group consists of liquefying pathogenic forms. This classification would exclude all gram-positive forms and spore-bearers, but not nonliquefying forms.

Klieneberger,¹ who studied strains derived from pathologic sources and from meat recommends the marked growth energy as the differential group characteristic and the putrefactive odor, peptonizing power and gram-staining reaction as characteristic of the different species. However, his study did not cover the different species described in the literature, and the characteristic of unusual growth energy as exhibited by rapidly spreading growth on agar, is not recorded of a number of species classified by other authors as proteus. This property is a characteristic of the species *vulgaris*, but not necessarily of others.

In the proposed classification of the committee of the Society of American Bacteriologists, *Proteus* Hauser may properly be considered as a genus under Family VI: "Bacteriaceae, rod-shaped organisms without endospores, gram-negative, flagella when present peritrichic, metabolism complex, amino-acids being utilized, and generally carbohydrates."

The proteus group may be considered to include those species which occur as rods varying from short coccoid forms to filaments, which are gram-negative, without endospores, flagella when present peritrichic, which are aerobes and facultative anaerobes, which liquefy gelatin, often producing characteristic stellate colonies, which often exhibit a marked rapidity of growth, which utilize amino-acids and generally carbohydrates which may be saprophytic or parasitic in their nature.

The type species of the proteus group may be considered to be the organism described in the literature as *Proteus vulgaris* Hauser. The type *Proteus mirabilis* first considered by Hauser to be a different species was later regarded by him (Hauser²) as an attenuated form of *vulgaris* and should probably be

considered identical with the latter. The third type, *Proteus zenkeri*, originally considered as a species distinct from *vulgaris* and later as an attenuated form of *vulgaris* is really a distinct species and inasmuch as it is unquestionably gram-positive and differs in all important points from *Proteus vulgaris*, it should not be included in the proteus group. It is probably identical with *B. zopfii*.

The characterization of *Proteus vulgaris* has been well established by the work of recent authors, including Berthelot, Horowitz, and others. It may be described as a gram-negative rod, which may exhibit pleomorphism, which is nonspore-forming, motile, noncapsulated, with peritrichic flagella, liquefying gelatin, often with the formation of characteristic colonies, fermenting dextrose with the formation of acid and gas, often fermenting saccharose, maltose and mannite but never lactose, precipitating then dissolving casein, producing a putrefactive odor from protein substances, usually forming indol, reducing nitrates and producing H_2S . In the present study rapidly spreading growth on agar, brown pigment production in broth, negative Voges-Proskauer reaction in peptone mediums, agglutination of related strains by immune serums, have been shown to be practically constant.

In addition to the type species *Proteus vulgaris* there are doubtless many more or less closely related forms which may be grouped as proteus species, as discussed by Jordan, which show the same fermentation reactions, but vary as regards proteolytic power as well as in certain other respects. A more complete study of such forms is needed to throw light on the relationship to *Proteus vulgaris* and other species of the proteus group.

An attempt has been made by a study of the literature to bring together species which have been classified under proteus and to determine as far as possible the validity of such classification. The earlier descriptions, however, are lacking in many of the essential points necessary to determine with accuracy the true nature of the organism in question and of its relation to other organisms classified in the same group. Such a classification must necessarily be considered tentative.

Genus *Proteus* Hauser

Proteus vulgaris Hauser

Syn. *Bacillus vulgaris* (Hauser) Mig. Macé.

Bacterium vulgare (Hauser) Lehmann-Neumann

B. vulgaris (Hauser) Chester

Bacillus proteus vulgaris Kruse

Identical or closely related forms

Proteus mirabilis (Hauser)

Syn. *B. mirabilis* (Hauser) Trev. Mig.

Proteus sulfureus Lindenborn, Holschewnikoff

Syn. *Bacillus sulfureus* Mig.

Bacillus murisepticus pleomorphus (Karlinski)

Syn. *Proteus* of Karlinski

Bacillus proteus anindologenes van Loghem and Loghem-Pouw

Proteus septicus Babes

Syn. *B. septicus* Babes Chester

B. proteus septicus (Babes) Kruse

Bacillus fetidus ozenae Hajek

Syn. *Bacillus ozenae* (Hajek)

Bacillus septicus putidus Roger

Bacillus ranicida Ernst

Bacillus proteus fluorescens Jaeger

Syn. *B. urinae* Chester

Pseudomonas jaegeri Mig.

Proteus fluorescens Macé

Proteus piscicidus versicolor Babes and Riegler

Syn. *Bac. piscicidus versicolor*

Bacillus cloacae Jordan

The following species do not conform to the description of the genus *Proteus* in one or more respects:

Proteus hominis capsulatus Bordoni-Uffreduzzi
Syn. *Proteus capsulatus septicus* Banti
 Bacterium proteus Migula
 Bacillus capsulatus septicus Kruse
Proteus zenkeri Hauser
Syn. *Bacillus zenkeri* Hauser
Bacillus zopfii Kurth
Syn. *Proteus zopfii*
 Bacterium zopfii
Proteus lethalis
Bacillus albus cadaveris (Strassmann-Strecker)
Syn. *Bacillus cadaveris* (Strassmann and Strecker)
Bacillus proteus ruber (Fortinau and Soubrauc)

Proteus mirabilis Hauser, first described as a separate species by Hauser,^{1,2} was later considered by this author and others an attenuated form of *Proteus vulgaris* and not a separate species.

Proteus sulfureus Lindenberg, Holschewnikoff as described by Holschewnikoff, is identical with *Proteus vulgaris* in morphology, pleomorphism, motility, nonspore-formation, appearance of colonies on gelatin, liquefaction of gelatin, appearance on agar and in milk, rapidity of growth, oxygen requirement. Formation of H_2S is emphasized, but *Proteus vulgaris* is known to produce large amounts of H_2S from peptone. The organism is described as occurring in water.

Proteus murisepticus pleomorphus Karlinski, an organism isolated from pus, as far as described by Karlinski, is apparently identical with *Proteus vulgaris*, occurring as a pleomorphic organism sometimes in the form of short rods, with rounded ends, sometimes as long rods and spirillum forms. The organisms were gram-negative and very motile. Spores were not observed. Gelatin colonies were surrounded by concentric rings and sinuous prolongations extending into the medium. Liquefaction of the medium followed with the emission of a butyric acidlike odor. Growth on agar was abundant and white, and broth showed a white sediment and emitted a strong odor. On blood serum growth was grayish white and thin, and the medium was rapidly liquefied. The organism was pathogenic for white mice, a loopful of a broth culture causing death in 24 hours, but gray mice were more resistant. *Proteus vulgaris* has often been shown to be pathogenic for mice.

Bacillus proteus anindologenes van Loghem and van Loghem-Pouw. Van Loghem and van Loghem-Pouw describe a number of organisms isolated by them from intestinal contents, feces, urine and intestinal abscess which varied from *Proteus vulgaris* in the nonproduction of indol. Berthelot has found that typical cultures which do not yield a positive indol reaction by the usual tests produce at least indol-3-acetic acid, and on this basis nonindol-producing strains should not be classified as a separate species.

Proteus septicus Babes.² This organism is described as a gram-negative, pleomorphic, motile bacillus, showing forms of varying lengths and 0.4 mikrons in width and without spores. A putrefactive odor was noticeable in cultures. The organism liquefied gelatin very energetically when first isolated, produced a spreading growth on agar and liquefied blood serum. The organism which killed mice and is described by the author as being very pathogenic was isolated from the blood and organs of a child dead of septicemia.

The author states that the organism is very similar to Hauser's *Proteus vulgaris*. The only discrepancy as far as described lies in the fact that the organism is stated to have stained gram-positively in the tissues.

Bacillus fetidus ozenae Hajek. The organism described by Hajek as occurring in the secretion of ozena coincides with *Proteus vulgaris* except in the matter of growth on gelatin plates, which is described as sometimes occurring with the formation of gas bubbles before liquefaction, which soon disappear, and the colony extends short projections into the medium, following which liquefaction takes place. The organism was a short gram-negative bacillus with a tendency to form in pairs or chains, was actively motile and nonspore-forming. Agar slants showed a slimy moist spreading growth outward from the line of inoculation, and an unpleasant putrefactive odor was noticeable. Blood serum showed a whitish growth covering the entire surface. Growth took place rapidly aerobically or anaerobically. The organism was pathogenic for mice, several drops of a gelatin culture injected subcutaneously causing death in 5 days.

Ward, who has recently made a study of the bacteriology of ozena isolated Perez' bacillus in 44% of 50 cases and proteus in 40%. Perez' bacillus according to this author is distinguished by slow fermentation of glucose, less luxuriant growth and less vigorous action on protein substances than *Proteus vulgaris* and a characteristic pigment in gelatin. Injections of Perez' bacillus and *Proteus vulgaris* in rabbits produced the same condition at necropsy, that is, increased nasal discharge and increase of temperature.

Bacillus septicus putidus Roger isolated from the liver and spinal fluid of a cholera patient who died with meningeal symptoms appears to be identical with *Proteus vulgaris* in staining properties, oxygen requirements, putrefactive odor, reactions in milk and carbohydrate mediums, liquefaction of gelatin and blood serum. Appearance of growth on agar is described as a thick, white, creamy growth along the line of inoculation, with semi-transparent separated colonies on the remainder of the surface. The author considers the organism as differing from *Proteus vulgaris* in not showing pleomorphism, in its more rapid liquefaction of gelatin, more marked turbidity in broth and different appearance on potato. The organism was not highly pathogenic, a rabbit receiving 1 c.c. intravenously succumbing after 2 or 3 days. He states the two organisms may be two different species or two strains of one species.

B. ranicida described by Ernst in a fish epidemic is stated by Babes and Riegler to have been indistinguishable from *Proteus vulgaris* except for a slight bluish opalescence on agar.

Bacillus proteus fluorescens Jaeger. This organism described by Jaeger as the cause of Weil's disease, was isolated from the urine during life, and from the blood and organs of patients dead of this disease. Though the etiology of this disease has been recently established on another basis, namely, that of *Spirocheta icterohemorrhagiae* as the causal agent, the isolation of a *Proteus vulgaris*-like organism from the urine has been reported by Pfaundler, Brüning, and others. Reports of the agglutination of the serum of patients suffering from this disease, with the typhoid bacillus are described by Eckhardt, Zupnik, Brüning and others, and of *B. typhosus* and *Proteus vulgaris* both by Lüdke.

The organism as described by Jaeger corresponds in general with *Proteus vulgaris* in its morphologic aspects, motility, lack of spores, staining properties, putrefactive odor and cultural behavior as far as recorded, except that growth on agar and in gelatin was characterized by a greenish fluorescence. Intraperitoneal or subcutaneous injection of mice caused death in a few days to two weeks. Migula classifies the organism as a *pseudomonas* though Jaeger describes peritrichic flagella. It seems proper to classify as a species in the proteus group.

Archibald has classified as *B. proteus fluorescens* an organism which he isolated from a case of choleraic diarrhea, which produced a greenish-yellow fluorescence on Drigalski-Conradi medium, which gave positive indol and Voges-Proskauer tests, produced acid and gas in dextrose, mannite, levulose, maltose, galactose and dextrin, but failed to ferment lactose, saccharose, dulcitate, adonite, inulin and raffinose, produced acid in milk, without curdling, then alkali, liquefied blood serum and was pathogenic for guinea-pigs.

Bataillon described an organism isolated in a fish epidemic which was distinguished from *Proteus vulgaris* by the production of a greenish sheen in gelatin.

Proteus piscicidus versicolor Babes and Riegler. This organism was described by Babes and Riegler in a fish epidemic and classed as a member of the proteus group, but differing from *Proteus vulgaris* in certain respects.

Colonies on agar were yellowish with thin transparent border. A noticeable putrefactive odor was produced. Growth was best at 20 C. Gas production was vigorous in sugar mediums. Characteristic proteus-like colonies were produced on gelatin plates. Glycerin potato showed a color display characteristic of the organism, the upper part being reddish-brown and the colors varying from top to bottom through brown, green, flesh-color and yellow. Gelatin stabs also showed similar color changes. Milk was coagulated with the formation of a brick-red scum. The organism was a gram-negative rod with peritrichic flagella. The organism was found to be pathogenic for fish, mice and rabbits. *Proteus vulgaris* which had also been isolated from the organs of the dead fish was found to be nonpathogenic for fish. The blood of the infected fish agglutinated the organism in dilutions of 1:50, but failed to agglutinate *Proteus vulgaris*.

Glaser and Hachla tested this organism for agglutination properties and found it to be agglutinated only by the strain used for immunizing, but not by serums obtained by immunizing with *Proteus vulgaris*.

Bacillus cloacae Jordan. This organism as described by Jordan is characterized by an inverted gas formula, namely, CO₂ in excess of H₂. In a number of strains isolated from water, all fermented dextrose and saccharose, and the majority lactose, though sometimes slowly. Most of the cultures liquefied gelatin, though some very slowly, and milk was acidified and curdled, the casein being dissolved in some cases. The indol reactions were variable. The organisms of this group were in general less actively proteolytic than *Proteus vulgaris*. Gas production was much more vigorous in dextrose than was the case with *B. coli*.

Levine classifies *B. cloacae* with *Aerogenes-cloacae* group under aerobic nonspore-forming bacteria which ferment lactose with gas formation, and describes *B. cloacae* as follows:

"Motile, gelatin liquefied (often very slowly); indol, dulcitol, glycerol, inulin and starch usually negative (rarely positive); dextrin occasionally positive; sucrose, raffinose, salicin and mannitol positive (rarely negative)." The group reactions include "Voges-Proskauer reaction positive; . . . reaction to methyl red alkaline, or if acid at first it reverts to a distinct alkaline reaction after long incubation (7 days); indol, usually negative, polysaccharids, starch, inulin and dextrin, negative or positive."

FOOTNOTE.—In Kruse's classification are included several organisms, *Bacillus b* (Vignal), *Bacillus havaniensis liquefaciens* (Sternberg), *Bacillus albus putidus* (Maschak) which as far as described may be included in the proteus group, while several others differ in regard to Gram's stain, non-liquefaction of gelatin and other respects, which would exclude them from this group. In his addenda to the proteus group Kruse includes pathogenic liquefying organisms, a number of which as far as described might also possibly be included in the proteus group: *Bacillus dysenteriae liquefaciens*, *Bacillus leucaemiae canis*, *Bacillus septicus ulceris gangraenosi* (Sternberg), *Bacillus pyogenes liquefaciens*, *Bacillus pyogenes gingivae* (Miller), *Bacillus pneumonicus agilis* (Flügge), *Bacillus leporis letalis* (Sternberg).

Glenn noted that *B. cloacae* differed from *Proteus vulgaris* in its ability to liquefy gelatin in the presence of carbohydrates, and concluded that the enzyme of proteus was more resistant to acids than that of *Proteus vulgaris*.

In this study Cultures 51 (from necropsied animal) and 68 (feces) correspond to the above descriptions of *B. cloacae*. They are characterized by very vigorous gas production in dextrose, saccharose, maltose and mannite, with less gas in raffinose and lactose in the case of 68 and with a large amount of gas in raffinose in the case of 51, but no gas in lactose in 5 days. Lactose was, however, fermented by this culture as indicated by a hydrogen-ion concentration represented by a P_H value of 5.4 in this medium. Neither culture liquefied gelatin in 3 days, but both showed a small amount of liquefaction in 30 days. Both yielded a positive Voges-Proskauer reaction and neither produced indol. Growth on agar was not spreading and the brown pigment production characteristic of *Proteus vulgaris* was absent. Milk was rendered acid and coagulated without peptonization.

Culture 45 has also been classified in this group, though not characteristic in all respects. Gas production was less vigorous in the carbohydrates above noted than was the case with Cultures 51 and 68. This culture presented one of the few cases of variation observed in the study. Two cultures of the same organism were tested as to carbohydrate reactions in the early part of the work, and gas production in lactose was recorded as a bubble, which in the case of one of the cultures disappeared in 7 days. In a test made over a year later, 15% of gas was recorded in lactose broth in 24 hours, and 20% in 7 days. This culture also presented the anomaly of fermenting dextrose much less actively than other carbohydrates. Liquefaction of gelatin by this culture took place more rapidly than in the case of Cultures 51 and 68. The Voges-Proskauer reaction was negative, differing in this respect also from 51 and 68.

The question as to whether *B. cloacae* should be classified in the proteus group or with *B. coli* has been considered by a number of authors. It is intermediate between the two, resembling *B. coli* in certain respects and *Proteus vulgaris* in others. The results obtained with the three cultures classified in this group illustrate the relationships to the above groups. Culture 45 in gelatin liquefaction, negative indol reaction, and negative Voges-Proskauer reaction seems more closely related to *Proteus vulgaris* than to *B. coli*. Culture 51 is identical with Culture 68 except that no gas was produced in lactose in 7 days, tho a certain amount of lactose was utilized as indicated by the hydrogen-ion concentration test. This culture on the basis of nonproduction of gas in lactose and liquefaction of gelatin would incline toward *Proteus vulgaris*, but it is undoubtedly very closely related to 68, which on the basis of gas production in lactose seems more like *B. coli*. A further study of liquefying lactose fermenters is needed to elucidate the relationship between these groups of organisms.

Proteus zenkeri Hauser

Syn. *Bacillus zenkeri* Hauser

Bacillus zopfii Kurth

Syn. *Bacterium zopfii*

Protens zopfii

The organism classified as *Proteus zenkeri* by Hauser¹ was originally described as a distinct species, but later was considered by this author and others as an attenuated form of *Proteus vulgaris* on the basis of positive gelatin liquefaction on long continued cultivation.

The organism is a thin rod, often appearing in the form of long filaments. Spore formation has not been observed. It retains the stain of Gram's method, is motile and shows characteristic growth on gelatin plates. The growth may be described as feathery or mycelium-like and occurs for the most part below the surface of the medium. No liquefaction of gelatin is reported in most of the descriptions. Growth on agar is a thin, transparent film. Macé describes a strong putrefactive odor in broth and negative indol formation.

B. zopfii (Kurth) is apparently identical with *Proteus zenkeri*. It is reported as found in long matted filaments or balls in old cultures. Klieneberger considers the two organisms identical on the basis of agglutination reactions.

In the cultural and agglutination tests made on the two members of this group, 73 *B. proteus zopfii* and 85 *Proteus zenkeri*, the behavior differed from that of *Proteus vulgaris* in nearly all respects. While the latter organism acts vigorously on carbohydrate and protein mediums, *B. proteus zopfii* and *Proteus zenkeri* were very inert. Growth on agar was nonspreading and showed filaments extending into the medium. Litmus milk was not curdled and showed no change of reaction or was very slightly alkaline after 14 days. Gelatin was not liquefied, tho the culture of *zopfii* was retained 4 months. No growth was obtained on Endo plates. No acid or gas production was observed in Russell's medium or other carbohydrate mediums. Indol production was negative, as was reduction of nitrates. A slight turbidity was produced in broth, but no pigment was noticeable. In the hanging drop the organisms, which are considerably larger than *Proteus vulgaris* and sometimes assume the form of long filaments, show a slight motility. Neither of the cultures was virulent for mice in 1 c c amounts.

In the tests with *Proteus vulgaris* immune serums no positive results were obtained except in the case of Serum 4 which agglutinated *zopfii* in a dilution of 0.01. *Proteus zenkeri* showed a tendency to agglutinate spontaneously throughout the tests.

The question of agglutination of *Proteus zenkeri* by human normal and typhoid serums was considered. Klieneberger¹ found both *B. proteus zopfii* and *Proteus zenkeri* agglutinated by human normal serums and therefore considers them identical.

The following results were obtained in my work with six serums tested:

		1:10	1:20	1:40
Normal serums:	7049	++++	+++	+
	7050	++++	++++	+++
	7054	++++	++++	++
Typhoid serums:	7051	++++	++++	++
	7052	++++	+++	++
	7053	++++	++++	+++
Control (no serum)		+		

The results obtained indicate that both normal and typhoid serums agglutinate *B. proteus zopfii* in dilutions of 1:40 or higher.

The two organisms which show identical reactions throughout and are apparently the same organism, are so distantly related to *Proteus vulgaris* that they should not properly be classified in the proteus group nor considered as attenuated forms of *Proteus vulgaris*. Gram-positive staining as well as cultural and agglutination reactions clearly differentiate them from the latter organism. It is recommended that these two forms be designated as *B. zopfii* and not considered as belonging to the proteus group.

The organism described by Bordoni-Uffreduzzi as *Proteus hominis capsulatus* in 3 cases of "Haderu-Krankheit," as *Proteus capsulatus septicus* by Banti, as *Bacillus capsulatus septicus* by Kruse should not properly be included in the *Proteus* group. The organism is described by Bordoni-Uffreduzzi as a gram-positive organism, showing a capsule in cultures. Gelatin was not liquefied. Macé calls attention to the resemblance of this organism to the pneumobacillus of Friedländer.

Proteus lethalis isolated by Babes² from the spleen and gangrenous lung of a patient who died of septicemia is described as a gram-positive, nonliquefying motile bacillus, pathogenic for mice and rabbits. This organism also is improperly included in the *proteus* group.

Bacillus albus cadaveris (Strassmann and Strecker) originally described as occurring in the blood of patients 4 days after death agrees with *Proteus vulgaris* in respect to appearance of gelatin colonies and gelatin liquefaction, putrefactive odor, nonspore-formation, active motility, fairly rapid growth on agar, pathogenicity for mice, but it is described as gram-positive. Whether this organism or the true *Proteus vulgaris* is a usual postmortem invader is apparently not established. Flexner, who isolated *Proteus vulgaris* in a case of peritonitis 12 hours after death, questions the frequent occurrence of *proteus*, since in his experience in routine necropsies *proteus* has been encountered rarely.

Bacillus proteus ruber isolated from river water by Fortineau and Soubrane is a gram-positive organism producing red growth on agar, and should probably not be included in the *proteus* group. The organism is described as liquefying gelatin slowly, and coagulating milk and as being pleomorphic in morphology.

SUMMARY AND CONCLUSIONS

The *proteus* group as described in the literature includes one well defined species, *Proteus vulgaris*, and a number of other species more or less distantly related, some of which clearly should not be thus classified.

As distinguishing characteristics of the group the following may be considered: Rods, varying from short coccoid forms to filaments, which are gram-negative, without endospores, with flagella when present, peritrichic, which are aerobes or facultative anaerobes, which liquefy gelatin, often producing characteristic stellate colonies, which utilize amino-acids and generally carbohydrates in their metabolism, which may be saprophytic or parasitic in their nature.

Proteus vulgaris may be described as a gram-negative rod, which may exhibit pleomorphism, which is nonspore-forming, motile, non-capsulated, with peritrichic flagella, which liquefies gelatin, often with the formation of characteristic colonies, which shows a rapidly spreading growth on agar, which ferments dextrose, with acid and gas formation, which often ferments saccharose, maltose, and mannite, but never lactose, which precipitates, then dissolves casein, which pro-

duces a putrefactive odor from protein substances, which usually produces indol and H_2S from peptone mediums, which reduces nitrates, which usually shows a negative Voges-Proskauer reaction in peptone mediums, which often produces brown pigment in mediums, which may be saprophytic or pathogenic, which is often agglutinated by immune serums derived from the same or related strains.

There are probably a number of related forms which are similar to *Proteus vulgaris* in fermentation reactions and in the property of liquefying gelatin, but which vary in proteolytic power and agglutination properties and other respects, which may be tentatively grouped as proteus species until further work may establish definite types.

A number of different species described in the literature may properly be considered as belonging to the genus proteus as listed under the heading Classification in this work. Several others, however, have been incorrectly classified here, including the organism designated as *Proteus zenkeri* which is a gram-positive organism and which differs in almost every particular from the other members of the group.

Ps. protea described by Frost, though closely related to *Proteus vulgaris* in cultural reactions, by reason of absence of peritrichic flagella would be unfortunately separated from the latter, in following the proposed classification based on morphologic characteristics. It is quite possible that other species classified as members of the proteus group do not possess peritrichic flagella, as descriptions are not sufficient to determine this point.

Proteus vulgaris possesses unusual agglutination properties. Somewhat discordant results have been obtained by different workers, but the results obtained in most of those in which true *Proteus vulgaris* has been considered, indicate that a relationship may often be established between different strains on the basis of agglutination reactions. It seems probable that strains from pathologic sources inter-agglutinate and that antibodies against the infecting organism are often produced, especially in suppurative processes, general infections and urinary infections. Such immunity seems to be lacking in cases of invasion of the digestive tract, however, except that a few cases of agglutination in low dilutions have been reported.

The results obtained in agglutination tests in this study point to the existence of a number of types. All of the strains derived from feces were agglutinated in comparatively high dilutions by a serum derived from a strain of fecal origin. This serum also agglutinated

in high dilutions 3 strains derived from water, 1 from meat, and several laboratory cultures of unknown history. An immune serum derived from a culture isolated from a wound bandage agglutinated the cultures derived from feces, but usually in lower dilutions than was the case with the preceding serum, and also agglutinated several cultures not agglutinated by the serum derived from the fecal culture. These included 1 from meat, 2 from necropsied animals and 1 from dog's saliva as well as 6 laboratory cultures. The results seem to indicate a type which is characteristic for cultures from fecal sources and another one perhaps from putrefactive sources outside of the body as well, as other types, though a final decision on the grouping cannot be established without further investigation.

Fermentation reactions did not necessarily correlate with the agglutination reactions in the tests made. It seems probable that closely related strains may sometimes vary either in respect to fermentation or agglutination reactions.

The agglutination of *Proteus vulgaris* cultures by the serum of patients with Weil's disease has been demonstrated only in low dilutions, and in some cases the organism cannot be identified with *Proteus vulgaris*. The agglutination of *Proteus vulgaris* by the serum of patients with typhus fever, on the other hand, is established on a better basis, agglutination by such serum having been reported as occurring regularly in high dilutions.

A number of cases are recorded of the agglutination of *B. typhosus* by the serum of patients with proteus infection, altho always in lower dilutions than with the homologous organism. Agglutination of proteus by the serum of patients with typhoid fever has been reported by some workers, while others have obtained negative results. In this work, a number of tests were carried out with serums which agglutinated *B. typhosus* in dilutions of 1:40 or higher, testing them against various cultures of *Proteus vulgaris* and with a culture of *Ps. protea*, which was found by Frost to be agglutinated by typhoid serums. Only one out of 13 cultures of *Proteus vulgaris* tested was regularly agglutinated by these serums, and this not as characteristically as was *B. typhosus* or the culture of *Ps. protea*. Agglutination of this culture was not evident after an incubation period of 1 hour, as was the case with *B. typhosus* and *Ps. protea*, but only after being retained at a temperature of 15 C. over night was there marked agglutination. One or two other proteus cultures were irregularly agglutinated by typhoid sera.

Proteus vulgaris is probably most frequently associated with decomposing organic matter of animal origin and the extent of its occurrence in water and soil probably bears a relation to the amount of such organic matter present. The occurrence of *proteus* in normal feces is apparently not as frequent as commonly stated.

Proteus vulgaris may be saprophytic or parasitic in nature. A pathogenic rôle has been ascribed to it by certain French workers in infantile diarrhea. *Proteus* has been associated with certain food poisoning epidemics as a possible causal agent, but such epidemics are relatively few in comparison with similar epidemics ascribed to *B. paratyphosus* B, and are not established on as firm a basis. As in the case of the latter organism the harmful effects produced may be due to the multiplication of the organism as well as to the formation of toxic substances, these toxins being very low in potency as contrasted with those produced by tetanus and diphtheria organisms.

A number of local infections including wound infections, in which *Proteus vulgaris* has been concerned as the primary agent or as secondary agent are described in the literature. Infections of the urinary tract due to this organism have been noted a number of times. Occasional general infections are also described.

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THE PRECIPITIN TEST FOR BLOOD IN FECES

LUDVIG HEKTOEN, BERNARD FANTUS

AND

SIDNEY A. PORTIS

From the John McCormick Institute for Infectious Diseases, Chicago

We have made a study of the precipitin test as applied to human feces. Fecal extracts were prepared in the following way:

Liquid feces were filtered directly by means of fine filter paper; semisolid or solid feces were mixed with 0.9% salt solution so as to obtain as concentrated an extract as possible and then filtered through a Buchner filter. If the extract was acid to litmus paper, it was neutralized with dilute sodium hydrate solution; if alkaline, by means of dilute hydrochloric acid. Chloroform was added to restrain bacterial growth. Clarification of the extract was secured by rapid centrifugation.

The precipitin tests were made in small, clear glass tubes, about 0.5 cm. in diameter; a small quantity of extract was placed in the tube and about 0.1 cm. of antihuman rabbit serum introduced at the bottom by means of a capillary pipet in such manner as to get a precise line of contact between the two fluids. The tubes were kept at room temperature and the results read after one hour. In most of the positive reactions there formed rather promptly a well defined precipitate in form of a grayish layer at the junction of the extract and the serum. As a rule the antihuman serum was 12,000 in titer, that is, it would give a precipitate within 20 minutes at room temperature with dilution of human blood 1:12,000 in salt solution. The specimens of feces from patients under treatment were obtained from the service of Dr. B. W. Sippy in the Presbyterian Hospital and from the private practice of Dr. Milton M. Portis; most of the specimens were obtained from the latter source. The specimens from cases of pernicious anemia came from patients in various hospitals. The specimens from normal persons were mostly from young men in military training, the others from medical students.

Our results are given in Table 1. In view of the fact that a positive result was obtained in such a large proportion of normal persons regardless of whether the Weber and benzidin tests were negative or positive, there is no necessity for any discussion of the possible value of the precipitin test in the diagnosis of occult blood in the feces. The only conceivable practical value of precipitin tests for human blood proteins in feces would be in cases giving a negative result with a precipitin test and a positive Weber or benzidin reaction. Under these circumstances a negative result with the precipitin test would indicate that the positive chemical test probably was not due to the presence of human blood.

The extracts of feces from normal persons on unrestricted diet were tested also with antibeeff, antishwine, antisheep and antichickens serums, but with practically uniformly negative results; in four instances only were not very strongly positive results noted, twice with antichickens serum and twice with antisheep serum.

TABLE 1
RESULTS OF PRECIPITIN TESTS OF FECAL EXTRACTS

Diagnosis		Weber Test		Benzidin Test		Precipitin Test (Antihuman Serum)			Remarks
Disease	No. of Cases	Negative	Positive	Negative	Positive	Negative	Positive	Per Cent. Posit.	
Normal.....	58	4	54	5	53	34	24	44	Unrestricted diet
Duodenal ulcer.....	6	4	2	5	1	2	4	66	Restricted diet; no meat
Gastric ulcer.....	4	..	4	2	2	..	4	100	Restricted diet; no meat
Cancer of esophagus	2	1	1	1	1	1	1	50	Restricted diet; no meat
Cancer of stomach..	2	..	2	..	2	1	1	50	Restricted diet; no meat
Colitis.....	11	9	2	9	2	4	7	63	Restricted diet; no meat
Cholecystitis.....	9	6	3	8	1	3	6	66	Restricted diet; no meat
Cardiorenal disease.	13	12	1	12	1	5	8	61	Restricted diet; no meat
Hyperthyroidism....	14	12	2	12	2	7	7	50	Restricted diet; no meat
Constipation.....	11	9	2	10	1	8	3	27	Restricted diet; no meat
Gastric catarrh.....	7	5	2	6	1	5	2	30	Restricted diet; no meat
Chronic appendicitis	4	4	..	3	1	2	2	50	Restricted diet; no meat
Nephritis.....	10	9	1	10	..	3	7	66	Restricted diet; no meat
Miscellaneous.....	34	20	14	27	7	20	14	41	Restricted diet; no meat
Pernicious anemia... (39 specimens)	16	31	8	33	6	3	36	92	Special diet and arsenic

These results are not in agreement with those of Kretowski,¹ in whose hands the precipitin test with antihuman serum of fecal extracts gave negative results when the chemical tests for blood were negative, and who regards a positive result with antihuman serum as direct evidence of the presence in the feces of human blood. Kretowski, however, does not say anything about the strength of the serum used and he examined altogether only a small number of cases. Citron² did not obtain any positive results with antihuman serum and extracts of normal feces, but he also omits to say anything about the strength of the serum used. In all the cases of intestinal disturbances which he examined, the feces being diarrheal, he obtained positive results. Our observations also indicate that liquid and semi-liquid feces give positive results with the precipitin tests more often than solid feces. The specimens examined from cases of pernicious anemia and which almost without exception gave positive results with the precipitin test were all or practically all liquid or semi-liquid.

¹ Nowiny Lekarskie, 1911, 23, p. 193.

² Arb. a. d. kais. Gesundheitsamte, 1911, 36, p. 358

At this time we are not concerned with the work of Brezina³ and others,⁴ who studied the precipitinogenic action of the contents of different parts of the intestinal tract. They did not study the action of antihuman serum on extracts of human feces; they did find, however, that among the antigenic substances in feces were such as would cause precipitins for the proteins of human blood to develop in the animals, but these antigenic substances were not at all present in large quantities. Kraus and Wilenko⁵ failed to get any reaction in cholera stools with antihuman serum which gave reaction with the stools of enteritis, tuberculosis and nephritis. From this fact they conclude that the cholera stool consists mostly of water and salts, and contains little or no proteins.

SUMMARY

Extracts of feces in salt solution often contain substances that form precipitate with antihuman serum. This seems to be the case just about as often in the case of healthy young men as of persons with various diseases. Such substances may be present in extracts of feces that do not give chemical tests for blood, and it may be inferred that such substances may be human proteins derived partly from the blood and partly also from the cells lining the intestinal tract.

The precipitin test for human blood in feces can be of only limited practical value; if negative in the presence of positive chemical blood tests, the indication would be that the blood is not human.

Extracts of feces of healthy men on unrestricted, full meat diet, only very exceptionally give positive reaction with antibeef, antisheep, antwine and antichickens serums, showing that in health foreign proteins taken into the stomach as a rule do not reach the feces as such.

In cases of pernicious anemia under treatment with arsenic the feces practically always give a positive reaction with antihuman serum.

³ Wien. klin. Wchnschr., 1907, 20, p. 560.

⁴ Brezina and Ranzi: Wien. klin. Wchnschr., 1908, 21, p. 1524. Solma and Wilenko: Ztschr. f. Immunitätsf., O., 1909, 3, p. 1.

⁵ Wien. klin. Wchnschr., 1909, 21, p. 150.

THE SPIROCHETE OF INFECTIOUS JAUNDICE (SPIROCHETA ICTEROHEMORRHAGIAE, INADA; LEPTOSPIRA, NOGUCHI) IN HOUSE RATS IN CHICAGO

ANDREW OTTERAAEN

From the John McCormick Institute for Infectious Diseases, Chicago.

In 1914 Inada and Ido¹ announced the discovery of a spirochete in the liver of a guinea-pig which had developed hemorrhagic jaundice and died as the result of the inoculation of blood from a case of infectious jaundice. This spirochete Inada later found to be the etiologic factor of Weil's disease and he named it *Spirocheta icterohemorrhagiae*. Due to certain characteristics, unlike all other spirochetes, Noguchi thinks it is a new genus and suggests the name *Leptospira*.

Miyajima,² in 1915, reported that he had found spirochetes resembling those described by Inada in the kidneys of field mice, and later that he had found, also in field mice, similar spirochetes, which when injected into guinea-pigs produced fever and hemorrhage, and after a number of generations, icterus. As immune serum against the spirochete of Inada was capable of destroying these organisms he concluded that the organisms in question were identical.

In 1917 Ido² and his co-workers observed two typical cases of spirochetosis icterohemorrhagica following the bite of rats, and were thus led to believe that the rat plays an important part in the transmission of the infection. On the basis of Miyajima's reports and of their own observations they undertook a series of experiments on house and roof rats in the city of Fukuoka and its vicinity in order to determine the prevalence of the spirochete in the rat. The kidneys of 92 rats were examined and spirochetes demonstrated in 26. In the urine from 71 rats spirochetes were present in 22. The blood and liver from 64 rats showed no spirochetes. Rats were also allowed to bite guinea-pigs in their legs, and of 50 experiments thus made, one

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¹ J. Exper. Med., 1916, 23, p. 377.

² J. Exper. Med., 1917, 26, p. 341.

guinea-pig died of icterus and hemorrhages on the 11th day after the bite. No spirochetes could, however, be demonstrated in the mouth of the rat. Altogether they found the organism in question in 40% of 149 rats examined. They also examined field mice and in one instance succeeded in infecting a guinea-pig by an intraperitoneal injection of kidney emulsion.

These results appear to have been confirmed by English and other European workers, who have recovered spirochetes both from the blood and urine of soldiers with hemorrhagic jaundice in France, and have also been able to demonstrate the organisms in the kidneys of field rats, and hemorrhagic jaundice in guinea-pigs. Stokes, Ryle and Tytler³ report that 5 rats of 9 taken from a certain section of a field, proved infective to guinea-pigs. From another section of the field 1 rat of 6 communicated the disease. Dawson, Hume, and Bedson⁴ report similar findings.

In the United States several workers report results similar to the Japanese and European. Noguchi⁵ found that rats captured in New York City and vicinity harbored the spirochete in question. Because he used an emulsion made from kidneys of several wild rats the exact percentage of rats communicating hemorrhagic jaundice to guinea-pigs is not known. Jobling, James, and Eggstein⁶ found that of a hundred rats captured in different parts of Nashville, Tenn., at least 10% carried the spirochetes and communicated the disease to guinea-pigs. Neill⁷ obtained a similar result from his experiments on wild rats in Washington, D.C.

In order to further extend the observations in regard to the prevalence of the causative organism of acute infectious jaundice in rats in the United States, experiments have been made on house rats captured in the basement of a hospital and of a medical college located in the City of Chicago. Thirty rats were used, a number of them being rather young. They were taken alive, killed, and one kidney aseptically removed at once. This kidney was then emulsified in salt solution, and the emulsion injected into the peritoneal cavity of a guinea-pig, one guinea-pig being used for each rat. The guinea-pig was then observed for at least 3 weeks, in most instances for 1 month.

³ *Lancet*, 1917, 192, p. 142.

⁴ *Brit. Med. Jour.*, 1917, 2, p. 345.

⁵ *J. Exper. Med.*, 1917, 25, p. 755.

⁶ *Jour. Am. Med. Assn.*, 1917, 69, p. 1787.

⁷ *Pub. Health Rep.*, May 10, 1918, p. 717.

The results of these observations were all negative. None of the guinea-pigs died, and at no time did they show any symptoms of hemorrhagic jaundice.

The other kidney together with the suprarenals, liver and spleen, the heart, lungs and the testicles were treated according to Levaditi's silver method and examined for spirochetes. A large number of individual sections from each organ were examined carefully, especial attention being given to the kidney, because it is in this organ that the spirochete has been found in the rat.

Of the tissues, the kidney from one rat (No. 15) was the only organ in which spirochetes were found. Here they were seen within epithelial cells of a convoluted tubule. In no other kidney were they found. And no spirochetes were found in other organs, namely, the heart, lungs, liver, spleen, suprarenals and testicles.

Films were also made from the heart blood, urine, kidney emulsion, and from swabs of the mouth and throat and scrapings from the teeth of the rats. The material from the mouth was examined because of the report that guinea-pigs, and in several instances human beings, who had been bitten by rats contracted hemorrhagic jaundice. It would therefore be of interest to know whether or not spirochetes are present in the mouth of the rat. This proved to be the case in one instance in my series. In rat 24 spirochetes were found in material taken from the mouth. In the films made from the urine, blood and kidney emulsion no spirochetes could be demonstrated.

The spirochete observed in the kidney of rat 15 was a very slender organism. In length it varied from about the diameter of a red cell to twice that length and longer. It had many rather closely coiled undulations which at times seemed to become smaller and more crowded toward the ends. These undulations were at times rather irregular and the ends were often hooked. Some of the spirochetes were placed in such a position toward each other as to resemble a branching organism. The spirochetes found in the material from the mouth of rat 24 were similar in shape to that just described except that, due to the staining method used, it was a little thicker. The morphology of the spirochetes observed by me answers to the description of the spirochete causing infectious jaundice.

In staining films for spirochetes previous experiments had proven that Tribondeau's modification of Fontana's method was rapid and gave a very satisfactory result, hence this method was used.

CONCLUSIONS

Spirochetes resembling those described as the cause of acute infectious jaundice were demonstrated in only two Chicago house rats of 30 examined (or 6.6%), indicating that the spirochetes probably are not present in a high percentage of such rats.

The spirochetes were demonstrated in material from the mouth and in tissue from the kidney.

Because of the presence of spirochetes in the mouth of rats it is possible that, at the time of the bite, organisms may be carried into the wound by the saliva or teeth, and disease in this manner transferred directly from the rat to man.

AGGLUTINATION OF STREPTOCOCCI

Y. NAKAYAMA

From the John McCormick Institute for Infectious Diseases, Chicago

Since many years different workers have tried to distinguish between strains of streptococci by means of agglutination, but so far uniform results have not been obtained. In 1902 F. Meyer¹ made experiments with the serum of animals immunized with streptococci from articular rheumatism, and he found that while nearly all the strains of streptococci of human origin which he examined were agglutinated by the serum, the strains from rheumatism and angina were agglutinated most readily while those from scarlet fever and suppurative processes were agglutinated in only slight degree if at all. On the basis of this result Meyer would classify streptococci into two groups; those from the pharynx, and those which are found in suppurative processes. Moser and von Pirquet² found that the serum of horses immunized with streptococci from scarlet fever would agglutinate such streptococci in high dilutions. Salge and Hasenknof³ observed that serum from patients recovering from scarlet fever would agglutinate streptococci from scarlet fever specifically, and had no effect on streptococci from other sources. These results seemed to indicate that the streptococci of scarlet fever were of a class by themselves. Jogichess,⁴ however, asserted that the agglutination of streptococci by scarlet fever serum was not specific. From extensive study of agglutination of streptococci by the serum of scarlet fever patients, Weaver⁵ concludes as follows: "Of streptococci cultivated from cases of scarlatina, some are agglutinated by almost all scarlatinal serum, but at dilutions varying from 1:160 to 1:4000; others are agglutinated by the same sera with less constancy and at lower dilution, and many are not agglutinated at all. . . . The agglutination reaction between the streptococci cultivated from cases of scarlatina and the serum from the cases of scarlet fever, is in no

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¹ Deutsch. med. Wchnschr., 1902, 28, p. 751.

² Centralbl. f. Bacteriol., I. O., 1903, 34, p. 714.

³ München med. Wchnschr., 1902, 49, p. 1729.

⁴ Centralbl. f. Bacteriol., I. O., 1904, 36, p. 692.

⁵ Jour. Infect. Dis., 1904, 1, p. 91.

way specific and cannot be of any value as a means of diagnosis." Aronson⁶ holds that while agglutination of streptococci is specific, changes occur in streptococci when they are passed through animals such as mice, rabbits, etc., the cocci acquiring new special characteristics. He immunized horses with streptococci from patients with pyemia, middle-ear infection, scarlet fever, and with streptococci from infections in horses, and found hardly any cross-agglutination between the serums thus obtained and the two groups of streptococci. Neufeld⁷ holds a still different view, and affirms that the agglutinability of streptococci and their toxicity are in inverse proportion, the stronger the toxicity of a given strain the less the agglutinability, and vice versa.

We see that there is no agreement among the investigators: Meyer distinguishes pyogenic streptococci from the pharyngeal by means of agglutination; Moser and von Pirquet, Salge and Hasenknof find a difference between streptococci from scarlet fever and streptococci from other sources, but Jogichess came to an opposite conclusion as did also Weaver; while Aronson asserts that streptococci readily change their agglutinative characteristics, and Neufeld lays stress on the fact that with increasing toxicity streptococci lose in agglutinability.

NEW EXPERIMENTS ON AGGLUTINATION

The following strains of streptococci were used in my experiments:

Strain	Source	Chains	Hemolysis	Growth in Dextrose Broth
1	Tonsillitis, scarlet fever	Long	+	Clear after 2-3 days
2	Tonsillitis, scarlet fever	Long	+	Cloudy
3	Tonsillitis, scarlet fever	Long	+	Cloudy
4	Tonsillitis, scarlet fever	Long	+	Cloudy
5	Pneumonia in horse	Long	0	Cloudy
6	Tonsillitis	Long	0	Cloudy
7	Frontal sinusitis	Long	+	Cloudy
8	Tonsillitis	Long	+	Clear
9	Abscess	Long	+	Cloudy

Rabbits were injected, first with streptococci grown in 2% dextrose broth for 24 hours, 1-3 loops of the centrifugated sediment being suspended in 3-5 c c of salt solution and then heated in the water-bath at 60 C. for 1 hour. Three more injections were given at intervals of 5-7 days of the same material in doses of 3-5 loops in 5-7 c c of salt solution, but without being heated. All injections were given intravenously, and serum was obtained 7-10 days after the last injection.

⁶ *Deutsch. med. Wchnschr.*, 1903, 29, p. 439.

⁷ *Ztschr. f. Hyg. u. Infektionskrankh.*, 1903, 44, p. 161.

The suspension of streptococci used in the tests consisted of cocci grown in 2% dextrose broth for 24 hours, suspended in salt solution after centrifugation, and shaking with small glass balls so as to break up the chains; 3% carbolic acid was added. The tests were made in capillary tubes; the mixtures were incubated at 37 C. for 2 hours and then left in room temperature for 24 hours before the results were read finally.

TABLE 1
AGGLUTINATION OF STREPTOCOCCI

Antiserums (Each serum is numbered the same as the streptococcus used in the immunization)	Streptococci								
	1	2	3	4	5	6	7	8	9
Antiserum 3.....	1,280	320	2,560	0 (1:40)	40	0	160	80	320
Antiserum 5.....	160	160	160	80	1,280	80	160	160	0
Antiserum 6.....	40	80	80	80	40	640	0	80	40
Antiserum 7.....	80	0	0	0	40	0	640	640	160

Table 1 gives the results obtained with the serum of rabbits immunized in the way described with strains 3, 5, 6 and 7. We note that three of the streptococci from scarlet fever are agglutinated in high dilutions by the serum of the rabbits immunized with a streptococcus strain from scarlet fever but not with the serum of rabbits immunized with streptococci from other sources; furthermore, that the serum of a rabbit immunized with the streptococcus from pneumonia in a horse agglutinated this streptococcus in higher dilution than it agglutinated streptococci from other sources; the same is also true with respect to streptococcus 6 and 7—in each case the homologous strain is agglutinated in higher dilutions than the strains from other sources. It should be noted, however, that the agglutination is not strictly specific because streptococcus 9, for instance, isolated from an abscess was agglutinated in a dilution of 1:320 of the serum of a rabbit immunized with streptococcus 3.

Special attention is directed to the fact that the serum obtained with streptococci 7 and 8 did not agglutinate streptococci from scarlet fever, and as this result seemed fairly specific absorption experiments were made in the following manner: Immune serum was diluted 10 times with 0.9% salt solution and 10 loopfuls were added of the centrifugated sediment of streptococcus cultures in 2% dextrose broth grown at 37 C. for 24 hours; this suspension was incubated for 2 hours and centrifugated thoroughly. With the clear fluid agglutination tests were made in the usual way. The results are shown in table 2. We

note that streptococcus 3 removes from its homologous serum the agglutinins for scarlatinal streptococci and to a less extent also the agglutinins for other streptococci. When the serum of rabbits immunized with streptococcus 1 and 2, also obtained from scarlet fever, was treated with streptococcus 3, the agglutinins for streptococcus 3 remain largely unaffected, showing that apparently there are differences in the agglutinins for different streptococci from scarlet fever. The agglutinins for streptococcus 3 are not removed by absorption with streptococcus 7, which was obtained from the frontal sinus. Streptococcus 7 removes the agglutinins from its antiserum for itself as well as for streptococcus 8 and 9. Streptococcus 9 removes the same agglutinins as does also streptococcus 8.

TABLE 2
ABSORPTION EXPERIMENTS WITH ANTISTREPTOCOCCUS SERUM

Treatment of Antiserum (Each antiserum is numbered the same as the streptococcus used in the immunization)	Streptococci								
	1	2	3	4	5	6	7	8	9
Antiserum 3 untreated...	1,280	320	2,560	0 (1:40)	40	0	160	80	320
Antiserum 3 treated with streptococcus 3.....	40	20	80	0 (1:20)	40	20	80	40	20
Antiserum 3 treated with streptococcus 7.....	640	320	2,560	0	40	20	0	80	80
Antiserum 3 treated with streptococcus 9.....	640	160	1,280	0	40	20	80	80	20
Antiserum 3 treated with streptococcus 1.....	40	80	160	0	40	20	80	20	80
Antiserum 3 treated with streptococcus 2.....	640	40	640	0	40	20	160	80	160
Antiserum 5 untreated...	160	160	160	80	1,280	80	160	160	0
Antiserum 5 treated with streptococcus 5.....	160	80	40	40	40	40	40	20	20
Antiserum 7 untreated...	80	0	0	0	40	0	640	640	160
Antiserum 7 treated with streptococcus 7.....	40	0	0	0	0	0	40	40	40
Antiserum 7 treated with streptococcus 9.....	80	0	0	0	0	0	160	160	0
Antiserum 7 treated with streptococcus 8.....	40	0	0	0	0	0	80	0	80
Antiserum 6 untreated...	40	80	80	80	40	640	0	80	40
Antiserum 6 treated with streptococcus 6.....	40	40	40	0	40	40	0	40	40

PHAGOCYTOSIS

The problem here was to determine whether streptococci could be separated into different groups by means of specific opsonins. I used the same serums as those in the agglutination experiments, and leukocytes from normal animals. The mixtures, equal parts, were incubated for 15 minutes when smears were made. The results are shown in table 3 and they appear to indicate that opsonins and agglutinins for

streptococci do not run parallel always because in some cases in which a serum agglutinated a given streptococcus it had no increased opsonic effect, and vice versa. The streptococci from scarlet fever appear to agree fairly well in their reactions with agglutinins and opsonins.

TABLE 3
RELATION OF AGGLUTININS TO OPSONINS IN ANTISTREPTOCOCCUS SERUMS

	Streptococci								
	1	2	3	4	5	6	7	8	9
Antiserum 3									
Opsonic index.....	2.6	2.9	3.9	1.7	1.2	0.8	2.6	1.4	0.95
Agglutination.....	1,280	320	2,560	0	40	0	160	80	320
Antiserum 5									
Opsonic index.....	1.3	0.8	1.2	2.7	2.0	1.2	2.8	1.33	1.9
Agglutination.....	160	160	160	80	1,280	80	160	160	0
Antiserum 6									
Opsonic index.....	1.35	1.3	2.2	1.85	1.15	3.1	0.9	2.1	1.3
Agglutination.....	40	80	80	80	40	640	0	80	40
Antiserum 7									
Opsonic index.....	1.0	1.3	1.1	1.4	0.93	1.35	2.0	1.5	1.5
Agglutination.....	80	0	0	0	40	0	640	640	160

0 indicates no result at 1:40.

AGGLUTINATION OF STREPTOCOCCI TREATED WITH CINNABAR

It has been shown that it is difficult to classify streptococci by means of agglutination because of the existence in the same anti-streptococcus serum of several distinct agglutinins. If the action of some of these agglutinins could be eliminated it might be easier to separate streptococci into different groups by means of agglutination. Perhaps this might be accomplished if streptococci could be rendered insusceptible to the action of such minor agglutinins. With this idea in mind I treated streptococci with cinnabar according to the method of Prof. T. Matsushita who suggested to me that by such treatment the reactions of streptococci by agglutinins might be modified.

Streptococci grown on blood agar for 24 hours at 37 C. were suspended in 0.9% salt solution to which a small amount of cinnabar was added; this mixture was then shaken for about 15 minutes and either centrifugated for a short time or allowed to stand for several hours until the cinnabar and other coarse particles had settled to the bottom, the supernatant fluid being used for immunization and for agglutination tests. For immunization with streptococci treated in this way I used streptococci 2 and 3, both from scarlet fever, and streptococcus 7, from the frontal sinuses. The results obtained in agglutination tests with the serums of rabbits immunized with cinnabar-

TABLE 4
COMPARISON OF AGGLUTINATION OF STREPTOCOCCI WITH AND WITHOUT TREATMENT
WITH CINNABAR

Strepto- coccus	Antiserum 3			Antiserum 7		
	Serum of Rabbit Immunized with Untreated Strepto- cocci + Untreated Strepto- cocci	Serum of Rabbit Immunized with Treated Strepto- cocci + Treated Strepto- cocci	Serum of Rabbit Immunized with Treated Strepto- cocci + Untreated Strepto- cocci	Serum of Rabbit Immunized with Untreated Strepto- cocci + Untreated Strepto- cocci	Serum of Rabbit Immunized with Treated Strepto- cocci + Treated Strepto- cocci	Serum of Rabbit Immunized with Treated Strepto- cocci + Untreated Strepto- cocci
3	2,560	2,560	2,560	—	—	—
1	1,280	80	640	—	—	—
2	320	40	80	—	—	—
7	160	40	40	640	5,120	5,120
8	—	—	—	640	160	320
9	320	160	320	160	1,280	1,280

TABLE 5
RESULTS OF TESTS WITH SERUMS PRODUCED WITH STREPTOCOCCI TREATED WITH CINNABAR
ACTING ON STREPTOCOCCI SO TREATED

Strain	Source	Chains	Hemol- ysis	Growth in Dextrose Broth	Agglutination		
					Anti- serum 3	Anti- serum 2	Anti- serum 7
1	Tonsillitis scarlet fever.....	Long	+	Clear after 2 or 3 days	80	640	160
2	Tonsillitis scarlet fever.....	Long	+	Cloudy	40	2,560	80
3	Tonsillitis scarlet fever.....	Long	+	Cloudy	2,560	320	80
4	Tonsillitis scarlet fever.....	Long	+	Cloudy	0	640	40
5	Pneumonia in horse.....	Long	0	Cloudy	40	—	160
6	Tonsillitis.....	Long	0	Cloudy	0	0	80
7	Frontal sinusitis.....	Long	+	Cloudy	40	40	5,120
8	Tonsillitis.....	Long	+	Clear	80	160	160
9	Abcess.....	Long	+	Cloudy	160	0	1,280
10	Abcess.....	Long	+	Cloudy	80	40	80
11	Tonsillitis.....	Long	+	Cloudy	640	—	80
12	Normal throat.....	Long	+	Cloudy	160	80	40
13	Chronic tonsillitis and pharyngitis.....	Long	+	Clear	160	80	80
14	Tonsillitis.....	Long	+	Cloudy	160	160	80
15	Hypertrophied tonsil.....	Long	+	Clear	80	—	160
16	Normal throat.....	Long	+	Cloudy	160	—	160
17	Tonsillitis.....	Long	+	Clear	160	0	320
18	Hypertrophy of tonsil.....	Long	+	Clear	80	40	640
19	Normal throat.....	Long	+	Clear	80	—	160
20	Tonsillitis.....	Long	+	Cloudy	320	80	320
21	Peritonsillar abscess.....	Long	+	Cloudy	320	160	40
22	Tonsillitis.....	Long	+	Cloudy	320	160	160

0 indicates no result at 1:40; — indicates no test

treated streptococci did not differ from those obtained with the serum of rabbits immunized with untreated streptococci so far as the homologous strains of streptococci are concerned, but in the case of heterologous strains of streptococci there was often less agglutination with the serum from the rabbits immunized with treated streptococci than with the serum of rabbits immunized with untreated streptococci. It appears that the treatment of streptococci with cinnabar in the way described presents to a certain degree the production of minor agglutinins when such streptococci are injected into rabbits (tables 4 and 5).

SUMMARY

It has been found that the serum of rabbits immunized with streptococci agglutinate homologous streptococci readily but heterologous streptococci less definitely.

It appears that there is considerable similarity on the part of streptococci obtained from scarlet fever, but it does not seem as if any distinct line can be drawn between streptococci obtained from the throat and ordinary suppurative processes by means of agglutination tests. Absorption experiments do not give decisive results.

When rabbits are immunized with streptococci treated with cinnabar the formation of minor agglutinins seems to be less than in rabbits immunized with streptococci that have not been so treated, but the results obtained from experiments along these lines are not definite enough to indicate that the method can be used to separate streptococci into distinct groups.

Agglutinins and opsonins do not always run parallel in the serum of rabbits immunized against streptococci, and it does not seem possible to classify streptococci by means of opsonins.

THE ORGANISMS OF SECONDARY INFECTION, ESPECIALLY PNEUMOCOCCI AND STREPTOCOCCI, IN PULMONARY TUBERCULOSIS

H. J. CORPER, W. G. DONALD, AND H. W. ANTZ

Laboratory of U. S. Army General Hospital, No. 16

Advances in other branches of medicine have always influenced and justified investigative work in tuberculosis. A true specific therapy for tuberculosis is still wanting, but it is well in the search for this not to neglect observations with a view to indirect beneficial influence on the course of the disease. It is for this reason that investigation of what might appear to be an exhausted field as indicated by the elaborate literature on "mixed" and "secondary" infections in pulmonary tuberculosis seemed justified. With the introduction of specific serum therapy for certain types of pneumococcus pneumonia it seemed important to obtain information on the applicability of such therapy in pulmonary tuberculosis; especially since some of the recent literature on secondary infections reports pneumococci in the blood of patients with pulmonary tuberculosis in as high as from 11-18% of the cases.

The discussion on "mixed" or "secondary" infection dates back almost to the time of the discovery of the tubercle bacillus by Koch and has finally resolved itself into three distinct schools originating from the different views held concerning the significance of such infection.

1. The lesions and more severe symptoms of pulmonary tuberculosis are always and only the result of mixed infection; a belief in the etiologic duality of the disease, held by the Koch school, Cornet, Petruschky and Spengler.

2. The lesions and symptoms in pulmonary tuberculosis may be caused by the tubercle bacillus alone, but not infrequently secondary organisms may contribute to the more severe symptoms or may be largely responsible for the unfavorable progress of the disease — Baldwin, Marmorek and Roemer.

3. Mixed infection exerts no influence on the true tuberculous process; a belief in the etiologic unity of the disease supported by v. Leyden, Straus, Fraenkel, Schroder, Mennes, etc.¹

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¹ For a more complete discussion and explanation of these conceptions see the article by Avery, O. T., and Lyall, H. W.: *Jour. Med. Research*, 1913, 28, p. 111; and Kogel, H.: *Internat. Centralbl. f. d. ges. Tuberk.-Forsch.*, 1913, p. 369.

To establish any criterion of the true significance of secondary infection in those organs or body cavities which have direct access to the air is admittedly exceedingly difficult. The secretions and excretions from these sources naturally contain numerous species of organisms which may simply vegetate in such a medium as saprophytes. We know today as a result of recent investigations especially of the poisonous gases, influenza, etc., that such saprophytes under ordinary circumstances are only potential, but may become kinetic under extraordinary circumstances, which may affect the normal individual as well as the consumptive. Clinical importance, therefore, immediately attaches itself to those bacteria found in the blood stream or in viscera having no direct communication with the air. There are certain well established facts, brought out by Avery and Lyall, showing that the tubercle bacillus alone can produce caseation, ulceration and cavity formation and with these pyrexia (Baldwin, Marmorek, Roemer), and that nontuberculous diseases of the respiratory tract have yielded a flora similar to that found in phthisis (Hastings and Niles), while the air passages of healthy individuals below the glottis are usually sterile (Krehl and Ritchie).

The results of examinations of the sputum and blood obtained by investigators from 1889-1912, in 1,083 cases during life and 685 cases postmortem, have been tabulated by Avery and Lyall and reveal the streptococcus, staphylococcus and pneumococcus as the predominating organisms in the sputum during life. These organisms likewise seem to be the most important ones found in the blood. It is noteworthy, however, that two sets of observers are entirely responsible for the high figures in the blood examinations, and in this respect their observations differ materially from those of the majority of previous and of two subsequent investigations. Pettit² in 1911 reports on 130 cases of pulmonary tuberculosis in which either the streptococcus (36 cases) or the pneumococcus (24 cases) was recovered in 60 cases, or 46%. Brown, Heise and Petroff³ in 1913, give the following positive figures in a final report on 157 cases of pulmonary tuberculosis: 9% of 33 incipient cases, 24% of 101 moderately advanced cases and 61% of 23 far advanced cases, a total of 26% in all. On the other hand, Avery and Lyall¹ in 1913 made bacteriologic studies of the blood in 115 cases of pulmonary tuberculosis in various stages of the disease and in 5 cases of bronchiectasis (in 20 of these cases the sputum findings were

² Trans. Natl. Assn. Study and Prevention Tuberculosis, 1911, 7, p. 310.

³ Ibid., 1913, 9, p. 344.

correlated with the blood findings). In no instance among the tuberculous or bronchiectatic cases was a secondary bacteremia demonstrated by blood culture. The sputum of the 5 cases of bronchiectasis and of the 15 cases of pulmonary tuberculosis revealed a flora differing little from that reported in a series of nontuberculous diseases of the respiratory tract. Harvey⁴ in 1916 reported on the examination of 22 specimens of sputum by a special method adapted to obtaining material from the tuberculous process itself and obtained results agreeing in the main with those of Avery and Lyall. Hall and Harvey⁵ examined the blood of 43 patients with pulmonary tuberculosis and obtained 2 positives (4.6%), one being positive over a period of 9 days, but not after a month, the other being negative after 5 days. They conclude that the methods of blood culture which give high percentages of positive results in the bacteremia of pneumonia and endocarditis are incapable of demonstrating a similar condition in pulmonary tuberculosis, even when the latter is presumably accompanied by severe infection.

The investigation now reported was carried on mainly with a view to applying the serum treatment of Cole of type I pneumococcus infections to consumptives, especially those presenting a bacteremia with this organism and incidentally to determine the relations between the micro-organisms found in the sputum and those found in the blood of the same individuals on a large series of cases to gain as many data as possible. The methods of sputum examination did not differ greatly from those used in investigations of recent years. We used only the ordinary washing methods for freeing the sputum from mouth bacteria and not the elaborate and time-consuming method tried by Harvey. The sputum after washing was then emulsified and streaked on human blood-agar plates previously tested for sterility. The streaks were made in such a manner that the majority of the organisms present would grow in individual pure colonies so that they could be used for further complete study and classification. Note was made of the predominating organisms besides considering all those present. Blood cultures were made at the time of the sputum examinations, but on account of the practical importance of the outcome the blood was studied much more elaborately aerobically and anaerobically. For aerobic study 1 and 5 c.c. were seeded into broth and incubated at

⁴ Jour. Med. Research, 1916, 35, p. 279.

⁵ Ibid., 1916, 35, p. 265.

least 72 hours, and 1 and 5 c c in agar at 43 C., which was immediately plated, several plates with each dilution of blood, and incubated at least 72 hours with frequent examination. Anaerobic studies were made using for this purpose the recently described Dick anaerobic plates,⁶ Wright's sodium hydroxid-pyrogallic acid method and an anaerobic paraffin sealed glass tube method which could be more easily

TABLE 1
SECONDARY ORGANISMS IN THE SPUTUM OF OPEN AND CLOSED CASES OF PULMONARY TUBERCULOSIS

Classifi- cation	Tuber- cle Bacilli	Pneumococcus Type				Streptococcus Type			Negative for Pneu- mocoeci or Strepto- coeci	Percent- age + Patho- genic Pneu. or Strep.
		I	II	III	IV	Hemo- lyticus	Viri- dans	Sapro- phyti- cus		
Incipient	+	0	3+1 ² + 1 ⁶	3+1 ² + 1 ⁷ +1 ⁹	5+1 ¹ + 1 ²	1	1	1+3 ¹	9+2 ¹ +3 ⁹	36
	—	0	1+2 ² + 1 ⁴ +1 ⁷	5+1 ³	5+1 ¹ + 2 ² +2 ³ + 1 ⁶ +3 ⁹ + 1 ¹⁰	0	7+1 ¹⁰	1+3 ¹ + 1 ¹⁰	17+2 ¹ + 1 ⁶ +1 ⁷ + 1 ⁸ +2 ⁹ + 1 ¹⁰ +1 ¹¹ + 1 ¹²	29
Moderately advanced	+	1	1+1 ²	5+1 ²	7	2	3	5 ¹	7+1 ¹ + 2 ⁷ +2 ⁹	42
	—	1	0	0	4+1 ¹	1	6+1 ⁹	4+2 ¹ + 2 ¹⁰	9+4 ¹ + 1 ⁶ +1 ⁹ + 1 ¹⁰	23
Far ad- vanced	+	0	3	1+1 ¹ + 2 ²	6+2 ² + 1 ⁶	1	0	2+1 ⁹	6+1 ¹ + 1 ¹⁰	27
	—	0	0	1	1+1 ²	0	1	0	3+1 ¹⁰	25

The numerals given (in large type) when totaled give the total number of cases in which the respective types of pneumococcus or streptococcus was found in the sputum. The column "negative for pneumocoeci or streptocoeci" gives the number of sputums in which no organisms of significance were isolated or other distinct organisms were isolated as indicated by the exponent.

The exponent indicates the organism isolated as tabulated below and when given under pneumococcus or streptococcus the respective organism was associated with one of these in the sputum. If the pneumococcus was present, the streptococcus associated was indicated by exponent: if the pneumococcus was absent and the streptococcus present, it was recorded under heading "Streptococcus Type."

The percentage figure given represents the percentage of pathogenic types of pneumococcus (I, II and III) and streptococcus (hemolyticus and viridans) as compared with the total number studied in each class (incipient +; incipient —; etc.).

Exponent 1: Staphylococcus

2: Streptococcus saprophyticus

3: Streptococcus viridans

4: Streptococcus hemolyticus

5: Streptococcus and M. catarrhalis

6: Micrococcus catarrhalis

Exponent 7: B. influenzae

8: B. pneumoniae (Friedländer)

9: Gram positive bacilli

10: Gram negative bacilli

11: Gram neg. b. and M. catarrhalis

12: Gram negative and positive bacilli

read. These were incubated 6 days. The utmost precautions were used in guaranteeing sterility of mediums (by preliminary incubation and numerous controls) and syringe (all glass Luer with slip-on needle was used) and skin (alcohol, ether and tincture of iodine). The sputums

⁶ Jour. Infect. Dis., 1918, 23, p. 578.

and bloods from 216 patients with pulmonary tuberculosis were examined; 104 of these were incipient cases, 76 moderately advanced, and 36 far advanced.

In order to note any differences in the sputum findings for bacteria besides the tubercle bacillus in open and closed cases of pulmonary tuberculosis, the 216 cases were classified on this basis with the results indicated in table 1. Since the most common organisms found in the sputum were pneumococci and streptococci and these are further typed, the table is divided so as to indicate the types found, the number under each type and the cases in which neither of these organisms were found. To make the report complete from a bacteriologic standpoint other organisms found alone or in conjunction with the former two are indicated by exponents.

TABLE 2
SECONDARY ORGANISMS IN THE SPUTUM OF TEMPERATURE POSITIVE AND TEMPERATURE
NEGATIVE CASES OF PULMONARY TUBERCULOSIS

Classifi- cation	Temp.	Pneumococcus Type				Streptococcus Type			Negative for Pneu- mococci or Strepto- cocci	Percent- age Patho- genic Pneu. or Strep.
		I	II	III	IV	Hemo- lyticus	Viri- dans	Sapro- phyti- cus		
Incipient	+	0	0	1	1+1 ⁹	0	0	1	1	20
	—	0	4+3 ² + 1 ⁴ +1 ⁶ + 1 ⁷	7+1 ² + 1 ³ +1 ⁷ + 1 ⁹	9+2 ¹ + 3 ² +2 ³ + 1 ⁶ +2 ⁹ + 1 ¹⁰	1	8+1 ¹⁰	1+6 ¹ + 1 ¹⁰	25+4 ¹ + 1 ⁶ +1 ⁷ + 1 ⁸ +5 ⁹ + 1 ¹⁰ +1 ¹¹ + 1 ¹²	30
Moderately advanced	+	0	1 ²	3	4+1 ¹	2	1	0	3+3 ¹ +2 ⁹	35
	—	2	1	2+1 ²	7	1	8+1 ⁹	4+7 ¹ + 2 ¹⁰	13+2 ¹ + 1 ⁶ +2 ⁷ + 1 ⁹ +1 ¹⁰	28
Far ad- vanced	+	0	3	1+2 ²	3+1 ²	1	0	0	4+1 ¹⁰	43
	—	0	0	1+1 ¹	4+2 ² + 1 ⁶	0	1	2+1 ⁹	5+1 ¹ + 1 ¹⁰	15

See notes for Table 1.

Study of table 1 reveals very little difference in the number or percentage of pathogenic pneumococci or streptococci as compared to nonpathogenic varieties found in the sputum of open or closed cases of pulmonary tuberculosis. Within the limit of error of the figures there is also noted no appreciable difference between the early and far-advanced cases.

In order to note any difference between febrile and nonfebrile cases, the sputum findings are classed accordingly in table 2.

It is noted that especially in the far-advanced cases the percentage of pathogenic varieties of organisms (especially pneumococci) is greater in the febrile than in the nonfebrile cases. In itself this result might be considered of significance, but what it means practically in the absence of other findings is not clear. If these findings were coupled, for instance, with similar findings in the early and moderately advanced cases, the value would be much more distinctly positive.

With a view to noting any difference in the sputum flora dependent on the presence of moisture in the lungs, the sputum findings have also been classified in table 3 on the basis of the absence and presence of râles.

TABLE 3
SECONDARY ORGANISMS IN THE SPUTUM OF CASES OF PULMONARY TUBERCULOSIS WITH AND WITHOUT RÂLES

Classification	Râles	Pneumococcus Type				Streptococcus Type			Negative for Pneumococci or Streptococci	Percentage Pathogenic Pneu. or Strep.
		I	II	III	IV	Hemolyticus	Viridans	Saprophyticus		
Incipient	+	0	$3+3^2+1^4+1^6+1^7$	$4+1^2+1^7+1^9$	$5+2^1+1^2+1^3+1^6+3^9+1^{10}$	1	$5+1^{10}$	$1+5^1+1^{10}$	$14+2^1+1^7+3^9+1^{11}$	35
	—	0	1	$4+1^3$	$5+2^2+1^3$	0	3	$1+1^1$	$12+2^1+1^6+1^8+2^9+1^{10}+1^{12}$	23
Moderately advanced	+	2	$1+1^2$	$5+1^2$	$11+1^1$	1	$8+1^9$	$4+6^1+1^{10}$	$15+3^1+2^7+2^9+1^{10}$	30
	—	0	0	0	0	2	1	1^1+1^{10}	$1+2^1+1^6+1^9$	30
Far advanced	+	0	3	$2+1^1+2^2$	$7+3^2+1^6$	1	1	2	$8+1^1+2^{10}$	29
	—	0	0	0	0	0	0	1^9	1	

See notes for Table 1.

Briefly, a comparison of the sputum findings on the basis of the presence or absence of râles reveals no appreciable differences in the findings considering the organisms from the standpoint of pathogenesis or the cases from the standpoint of amount of anatomic involvement.

Blood cultures made during life and many repeated several times (some as many as 4 or 5 times) gave consistently negative results.

The blood culture results in the 216 cases were positive 7 times, but these positives were only obtained in the broth cultures, never in the solid mediums, aerobic or anaerobic. Of these 7, four gave

staphylococcus, which makes it highly probable that they were skin contaminators and should be excluded. The other positives reveal a striking irregularity in the coincident findings, that is, sputum and blood findings — and absence or presence of fever. Considering these positives, the percentage agrees well with those of Hall and Harvey — repetition of results by subsequent blood culture, with 3-5 days, however, were not obtained.

TABLE 4
BLOOD CULTURES DURING LIFE

Classification	Total Number Examined	Positive Findings†	Tubercle Bacilli in Sputum	Other Organisms in Sputum	Temperature	Râles	Percentage Positive Blood Cultures
Incipient	104	1 case Staphylococcus* aureus	—	B. pneumoniae (Friedländer)	—	—	0.0 *
Moderately advanced	76	2 { 1 case Staphylococcus aureus 1 case Micrococcus tetragenous	— +	Streptococcus saprophyticus (Both)	— (Both)	+ (Both)	1.3
Far advanced	36	1 Pneumococcus, type II 1 Staphylococcus aureus 1 Streptococcus + Pneumococcus, type IV 1 Staphylococcus albus	+ + + +	Pneumococcus IV Pneumococcus IV Negative Streptococcus saprophyticus	+ — — —	+ + + +	5.5

* Staphylococcus was found in broth culture only not in plates. Staphylococci were not included in the positive percentage figures.

† All positive blood results were obtained in broth cultures only — never in plate cultures aerobic or anaerobic. Controls in which small numbers (10 to 20) virulent pneumococci and hemolytic streptococci were added to blood studied for control on liquid and solid mediums always gave positive results (numerous individual colonies on plates within 24 to 48 hours).

In the series of 216 cases studied there were 8 deaths within one month of the time of the sputum examination, which offered opportunity for necropsy and blood culture studies. The results are recorded in table 5.

Of the 8 cases examined ante- and postmortem, the blood of one of the cases revealed a pneumococcus type 2 three weeks before death, but was negative subsequently and at postmortem; one of the cases which was negative one week before death revealed a pneumococcus type II in the lung and blood postmortem. (It is noteworthy, that all postmortem blood cultures were made within 4-10 hours after death, and that immediately after death the body was placed in a refrigerator below freezing point.) The rest of the cases (6), were negative, both before and after death.

It appears from the results of these investigations that secondary organisms (especially pneumococci and streptococci), found in the sputum of cases of pulmonary tuberculosis ordinarily play an insignificant part in the pathogenesis of this disease. This conclusion seems especially justified when the high percentage tendency (50%) of pneumococci and streptococci to enter the circulation as evidenced by positive blood cultures in pneumonias due to these organisms is

TABLE 5

RESULTS OF ANTEMORTEM SPUTUM AND ANTE- AND POSTMORTEM BLOOD CULTURES IN CASES OF PULMONARY TUBERCULOSIS

Case	Brief Anatomic Diagnosis	Sputum Culture Ante-mortem	Blood Culture Ante-mortem	Blood Culture Post-mortem*
1	Ulcerative pulmonary tuberculosis, left spontaneous pneumothorax, ulcerative enteritis, tuberculous adenitis	Pneumococcus II, Streptococcus saprophyticus	— one month before death	—
2	Chronic ulcerative pulmonary tuberculosis, ulcerative enteritis, and tuberculous adenitis	Pneumococcus II	— one week before death	+ Pneumococcus II†
3	Ulcerative pulmonary tuberculosis, pyo-pneumothorax, tuberculous adenitis	Pneumococcus IV	+ Pneu. II 3 weeks before — one week before death — 4½ hours before death	—
4	Ulcerative pulmonary tuberculosis, left spontaneous pneumothorax, tuberculous enteritis and adenitis	Negative for pathogens	— two weeks before death	—
5	Ulcerative pulmonary tuberculosis, ulcerative tuberculous enteritis, tuberculous adenitis, syphilitis aortitis	Gram negative bacillus	— one week before death	—
6	Ulcerative pulmonary tuberculosis, ulcerative enteritis and appendicitis, tuberculous laryngitis	Pneumococcus III, Streptococcus saprophyticus	— one month before death	—
7	Ulcerative pulmonary tuberculosis, unresolved influenzal bronchopneumonia, ulcerative enteritis, tuberculous adenitis	Streptococcus hemolyticus	— three weeks before death	—
8	Ulcerative pulmonary tuberculosis, ulcerative enteritis, pericarditis with effusion, adenoma of liver	Pneumococcus II-a	— one month before death	—

* All postmortem examinations were made within 4-10 hours after death, the bodies having been kept at freezing temperature during this time.

† Lung postmortem showed the pneumococcus, type II.

considered (the pneumococcus was found in the blood in as high as 40% of the severe influenzal bronchopneumonia cases studied during the recent epidemic by the authors), and the complete absence even of the pathogenic variety of these organisms in the blood of cases of pulmonary tuberculosis. As far as the presence of these secondary organisms in the lung are concerned, these observations tend to support the contention that the susceptibility of the consumptive to secondary hematogenous infection is to be viewed more in the light of a case of nontuberculous disease of the lung with organisms present in the lungs producing no particular destructive changes ordinarily. These organ-

isms can, however, play a definite, but distinct part in the pulmonary changes when conditions arise such as occurred during the influenza epidemic, when tuberculosis and influenza were found as two distinct conditions in the lungs, but in this the consumptive does not differ materially from the normal individual inasmuch as these are two separate infections. However, it must be appreciated that there is a lowered local resistance in the lung of the consumptive over normal individuals that makes possible the presence of secondary organisms in the lungs thus resembling the condition in the case of nontuberculous pulmonary disease, but tuberculosis per se is not accountable for the presence of these organisms in the circulating blood.

SUMMARY

The examination of the blood of 216 cases of pulmonary tuberculosis (104 incipient, 76 moderately advanced and 36 far advanced) using for this purpose both liquid and solid mediums (aerobic and anaerobic), resulted in growth of bacteria in broth only, not in solid mediums, in 7 of the cases, 4 of which gave staphylococcus, a possible skin contaminator, one a moderately advanced case, micrococcus tetragenous, and two far-advanced cases gave one pneumococcus type 2 and one pneumococcus type 4 and streptococcus saprophyticus. Eight of the 216 cases examined died within a month after such examination, revealing ulcerative pulmonary tuberculosis, and only one of these gave a positive culture after death (type 2 pneumococcus in blood and lungs), whereas blood culture one week before death was negative. Another case in which blood culture was positive 3 weeks before death was negative postmortem.

Sputum cultures made coincidently with blood examination to correlate the results revealed in the majority of cases pneumococci and streptococci. The most common pathogenic varieties being pneumococci—types I and III—and *Streptococcus viridans*. A few pneumococci—type I—and hemolytic streptococci were found. Approximately 30% of the sputums revealed pathogenic pneumococci and streptococci. No practical differences were noted in the percentage of pathogenic pneumococci and streptococci found when the cases were classified according to open or closed tuberculosis, with or without fever, and with or without râles.

ANAPHYLATOXIN AND ANAPHYLAXIS
XI. ULTRA-FILTRATION AND FRACTIONATION OF
ANAPHYLATOXIN *

PAUL H. DE KRUIF AND ARNOLD H. EGGERTH

From the Hygienic Laboratory of the University of Michigan, Ann Arbor

SYNOPSIS

INTRODUCTION.

THE FRACTIONATION OF ANAPHYLATOXIC SERUMS

BY DIALYSIS.

BY DILUTION AND ACIDIFICATION.

PRECIPITATION OF ANAPHYLATOXIC PROTEIN BY THE METHOD OF HARDY
AND GARDINER.

THE ULTRA-FILTRATION OF ANAPHYLATOXIN.

THE FRACTIONATION OF TOXIC NORMAL AND IMMUNE SERUMS.

SUMMARY.

Despite the large amount of study on the nature of anaphylatoxin and of the mechanism of its formation, little if any attempt has been made to isolate the toxic principle on one or the other of the protein fractions of the serum. Various serum principles having physiologic activity, but of unknown chemical nature, have been isolated on the protein fractions of serum. The association of these active principles is often so close as to suggest that they may themselves be part of one of the serum proteins. Thus Alex. Schmidt,¹ by diluting and passing CO₂ through blood plasma precipitated a "fibrinoplastic" substance which was able to clot hydrocele fluid. Seng² quantitatively isolated diphtheria antitoxin on the water-soluble globulin of immune serum. Fuld and Spiro³ found that the rennin-inhibiting activity of normal serum is associated with the water-soluble globulin, while the water-insoluble globulin on the other hand has a distinct milk coagulating action. Pick⁴ made an extensive study of the various immunity principles of serum and found antitoxins, lysins and agglutinins to be associated with one or the other globulin fractions. The work of

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* For Parts I-X, see this Journal, 1917, 20, p. 499; also, Jour. Am. Med. Assn., 1917, 68, p. 1524.

¹ Arch. f. Anat., Physiol., u. wissenschaft. Med., 1862, p. 461.

² Ztschr. f. Hyg. u. Infektionskrankh., 1899, 31, p. 513.

³ Ztschr. f. physiol. Chem., 1900, 31, p. 140.

⁴ Beitr. z. chem. Phys. u. Path., 1902, 1, p. 351.

this author and of Seng has been practically applied to the concentration of antitoxins by Gibson,⁵ Banzhaf⁶ and others.

While it is entirely possible that these active principles of the serum may be relatively simple substances, attached by adsorption or otherwise to the serum proteins, the other possibility exists that the proteins themselves may be physiologically active. The fact remains that up till now no clear evidence of immune bodies freed entirely from serum proteins has been presented, and such high authority as Landsteiner⁷ thinks that these substances may actually be albuminoid in character.

It would be of great interest to determine whether the anaphylatoxic principle can be isolated on serum proteins. Such procedure, if possible, would not only furnish an approach to the discovery of the nature of the toxic principle, but might eventually throw light on the mechanism of its formation. We have, accordingly, applied various methods of fractionation of serum proteins to this investigation and in addition to this have attempted to locate the toxic principle by more recently devised physico-chemical methods.

Before entering into the experimental part, it would be well to outline very briefly the prevailing notions of the number and nature of the serum proteins and definitely to define the meaning of the terms "euglobulin," "pseudoglobulin," etc., frequently used in this paper.

The water-insoluble globulin of serum was first thoroughly studied by Panum.⁸ He obtained this protein by simple dilution, or by dilution with subsequent passage of CO₂ or addition of acetic acid. This author gave to the water-insoluble globulin the name "serum-casein." Kühne⁹ failed to find casein characteristics in this substance and gave it the name "paraglobulin." Heynsius¹⁰ found that this paraglobulin, besides being obtained by the methods just mentioned, could be separated by the saturation of serum with NaCl. Aronstein¹¹ was the first to show that the paraglobulin could be flocked out by dialysis of serum against distilled water. These dialysis studies were confirmed and extended by Alex. Schmidt.¹²

⁵ Jour. Biol. Chem., 1906, 1, p. 161.

⁶ Med. Rec., New York, 1909, 75, p. 581; Collected Studies from the Research Laboratory, Department of Health, City of N. Y., 1911, p. 153.

⁷ Kolle u. Wassermann Handb. d. path. Mikro-org., 1913, 2, p. 1241.

⁸ Virchow's Arch. f. path. Anat., 1852, 4, p. 23.

⁹ Kühne's Lehrbuch der physiol. Chemie, 1868, p. 168, cited from Hammarsten, Erg. d. Physiol., 1, part 1, p. 333.

¹⁰ Pflüger's Arch. f. Physiol., 1869, 2, p. 1.

¹¹ Pflüger's Arch. f. Physiol., 1874, 8, p. 75.

¹² Beitr. d. Anat. u. Physiol. als Festgabe Ludwig gewidmet., 1874.

Hammarsten¹³ was led to believe that none of the methods alluded to completely separated the paraglobulin from serum, but that such separation could be achieved by a method long ago described by Denis.¹⁴ This procedure consisted simply in saturating serum with MgSO_4 . Hammarsten thought that all the globulin precipitated by this method was of the same nature. This idea was disputed by Burckhardt,¹⁵ who claimed that the protein thrown out by saturation with MgSO_4 was not one substance, but could be separated into water soluble and insoluble portions. Hammarsten objected to this on various grounds, but many other workers brought confirmation to the findings of Burckhardt.

Kauder,¹⁶ making use of the discovery of Méhu that $(\text{NH}_4)_2\text{SO}_4$ would precipitate substances of albuminoid nature, studied the fractional precipitation of serum proteins by this reagent. This investigator found that while it required full saturation with MgSO_4 to precipitate serum globulin, $(\text{NH}_4)_2\text{SO}_4$ had only to be added to half-saturation to accomplish the same result. In 1900, Fuld and Spiro,³ during a study of the rennin-like and rennin-inhibiting substance of serum, made an extensive investigation of the $(\text{NH}_4)_2\text{SO}_4$ fractionation of serum proteins. They divided the total globulin secured by half-saturation with this reagent into two bodies; one, which they designated "euglobulin" precipitating between 28-33% saturation and water-insoluble, and the other, flocking out between 34-46%, water-soluble, which they named "pseudoglobulin." This terminology has been widely applied to the serum proteins. The majority of workers refer to three serum proteins—euglobulin, pseudoglobulin and albumin..

Two objections have been made to this classification. One, by Freund and Joachim,¹⁷ who claim that there are in reality no fewer than six globulin bodies, and that these can be separated by $(\text{NH}_4)_2\text{SO}_4$ fractionation. These authors state the various globulins differ in certain physical constants, particularly in heat coagulation temperature. But it has been shown by Haslam¹⁸ that the salting out of proteins from a mixture must be carried out in the same way as the separation of mixtures of fluid substances by fractional distillation, and that it frequently requires as many as 17 reprecipitations to obtain

¹³ Pflüger's Arch. f. Physiol., 1878, 17, p. 413.

¹⁴ Mémoire sur le Sang, 1859.

¹⁵ Arch. f. exper. Path. u. Pharmacol., 1883, 16, p. 322.

¹⁶ Arch. f. exper. Path. u. Pharmacol., 1886, 20, p. 415.

¹⁷ Ztschr. f. physiol. Chem., 1902, 36, p. 407.

¹⁸ Biochem. Jour., 1913, 7, p. 492.

pure products of the protein fractions. The numerous globulins of Freund and Joachim, then, are in reality due to incomplete separation of mixtures of the protein fractions. The marked differences in heat-coagulation temperature are not significant in their work because they did not rigidly control the acidity, salt concentration, and other factors which the work of Chick and Martin and others has shown to be highly important.

The second objection to the classification of Fuld and Spiro is made by some authors who think that there is in reality only one serum globulin. Space does not permit us to go into these objections at length, but the important work of Chick¹⁹ merits mention in this connection. This investigator believes that only two proteins, globulin and albumin, exist in the serum. The apparent difference in water solubility of parts of the globulin is due to the formation of an adsorption complex between a part of the globulin and lecithin. This complex is water-insoluble and has the properties of euglobulin. Chick was able by addition of weak lecithin emulsions to globulin containing no phosphorus to obtain a product having in general the characteristics of euglobulin. What is more, by long continued dialysis at a slightly raised temperature, Chick was able to change all the globulin of serum to a water-insoluble condition. This is due, in her opinion, to a gradual denaturation of the water-soluble globulin with the simultaneous formation of the lipoid complex. The finding of Hardy²⁰ that the euglobulin contained a definite amount of phosphorus while the pseudoglobulin possessed only traces of this substance is referred by Chick to the failure to disrupt the lecithin-globulin complex. In this connection Chick points to the work of Hartley,²¹ who studied the constitution of serum protein by the Van Slyke nitrous acid method, and who could find chemical differences between the whole globulin and albumin only.

It is quite possible that Chick is right in her contention and that only one globulin does exist in the serum. However, for practical purposes it is convenient to distinguish between water-insoluble and water-soluble globulin bodies. Whether euglobulin is only a complex of water-soluble globulin with lecithin or not, the fact remains that a certain quite definite amount of this substance comes down on dialysis, on dilution + acidification and on NaCl saturation, while another por-

¹⁹ *Biochem. Jour.*, 1914, 8, p. 404.

²⁰ *Jour. Physiol.*, 1905, 33, p. 251.

²¹ *Biochem. Jour.*, 1914, 8, p. 541.

tion remains in solution. Bearing in mind that the distinction may be an unreal one, we will speak of three types of protein in the following work—the total globulin, separated from the albumin by half-saturation with $(\text{NH}_4)_2\text{SO}_4$, and in turn divided into a water soluble and insoluble portion by the procedures described in the preceding sentence.

DIALYSIS OF ANAPHYLATOXIN

The method of dialysis was first attempted, and was conducted in collodion sacs prepared by the Novy-Gorsline²² method. These sacs were found to be greatly superior to the parchment bags ordinarily used. The first experiment was made to determine whether any part of the anaphylatoxic principle of rat serum could be isolated on the euglobulin obtained by dialysis.

The method used for the production of anaphylatoxin, whether from rat or guinea-pig serum, was the "sol-gel" agar method described by Novy and De Kruif.²³ We will describe this briefly at this point, and thereafter confine ourselves to the statement that 'the anaphylatoxin was prepared in the usual manner,' unless some deviation from this technic was resorted to.

Six rats were bled from the heart, the blood pooled and centrifugated at 8,000 revolutions. Fifteen cc of the supernatant serum were mixed with 3.75 cc of agar (0.5%) hydrosol which had been previously kept at a temperature of 37 C. for 1 hour. The mixture was shaken thoroughly, placed in cracked ice for 2 hours, and following this incubated for 30 minutes. The agar was removed by centrifugation and the resulting supernatant fluid tested for toxicity. The result is given in A of Table 1.

Four cc of the anaphylatoxin were placed in a thin collodion sac and dialyzed against running distilled water for 22 hours. The heavy precipitate of euglobulin was removed by centrifugation at 3,000 revolutions.

The supernatant fluid, B, from this centrifugation was made isotonic with 17% NaCl and found to have increased in volume to an extent that 6.15 cc of this fluid were equivalent to 4 cc of the anaphylatoxin before dialysis. The toxicity of fluid B was tested by intravenous injection into guinea-pigs. The result is given in B of Table 1.

The euglobulin precipitate, C, removed from the dialysee by the centrifugation just described, was dissolved in 1 cc of 0.85% NaCl solution. The solution, which was very opalescent, was injected and the result is recorded in C of Table 1.

The experiment just recorded makes no attempt at a quantitative estimation of the amount of toxic principle recovered, but indicates

²² Contrib. to Med. Research, dedicated to V. C. Vaughan, 1903, p. 390.

²³ Jour. Infect. Dis., 1917, 20, p. 536.

merely that all of the active principle is not inactivated by dialysis, and moreover that it is present on both the water-soluble and insoluble fractions. An error was made in this experiment which we attempted afterward to avoid — the use of too little NaCl solution in dissolving the euglobulin precipitate. The solution obtained in this experiment was of a very low grade of dispersion, and many trials made subsequently have convinced us that a highly dispersed solution is necessary to demonstrate all of the toxic principle carried down with the euglobulin fraction.

TABLE 1
THE DIALYSIS OF RAT ANAPHYLATOXIN

Solution	Guinea-Pig		C C Intra- venously	Serum Equiva- lent	Result
	No.	Weight			
A (Anaphylatoxin).....	1	200	0.25	0.25	Nil
	2	210	0.5	0.5	+3'55"
B (Pseudoglobulin + albumin)....	3	200	6.15	4.0	+4'30"*
C (Euglobulin).....	4	210	1.0	4.0	+3'30"*

* The pictures of shock, the method of death, and the necropsy findings in the guinea-pigs dying from the injections of B and C correspond perfectly with that from the original anaphylatoxin A.

The euglobulin recovered from dialysis of normal rat serum was tested for toxicity as a control, and it was found that 5, 8 and even 10 c c serum equivalent of this substance produced slight if any toxic effect when injected intravenously into guinea-pigs of 200 gm. weight. Rat serum, like that of many other animal species, shows varying degrees of primary toxicity, but this toxicity has in no case been found to precipitate with the water-insoluble globulin.

Many dialyses of rat anaphylatoxin were carried out with disappointingly irregular results. Given samples of anaphylatoxin were in several instances divided into 3 or 4 equal parts, placed in collodion sacs of as nearly as possible like permeability, and dialyzed for identical lengths of time. In spite of these precautions it frequently happened that the euglobulin in one of the sacs would prove lethal to guinea-pigs while that from the others had no effect.

It appeared from any tests by dialysis that the toxic principle did not separate out exclusively on one fraction, but that a partition on the water-soluble and insoluble fractions took place. The following experiment indicates this fact clearly.

Rat anaphylatoxin prepared from rat serum by the usual sol-gel method was tested for toxicity and found to be fatal to guinea-pigs of 200 gm. weight in dose of 0.25 c.c. Ten c.c of this were placed in a thin collodion sac and dialyzed against running distilled water for 24 hours.

A. The precipitate was centrifugated out at 8,000 revolutions and dissolved in 10 c.c of 0.85% NaCl (original volume). The solution, which was very opalescent, was cleared by the addition before injection of a few drops of $N/25$ Na_2CO_3 . The result of the injections is given in A, Table 2.

B. After removal of the euglobulin precipitate A, CO_2 was bubbled through the supernatant and resulted in a further gummy precipitate, which was dissolved in NaCl solution as in the case of A, and cleared with alkali. The result of the injections is given in B, Table 2.

C. The supernatant remaining after the passage of the CO_2 was concentrated to 7.6 c.c in a current of warm air and made isotonic with the addition of 0.4 c.c of 17% NaCl solution. Result of injections is given in C, Table 2.

TABLE 2
DIALYSIS OF RAT ANAPHYLATOXIN

Solution	Guinea-Pig		C C Intra- venously	Serum Equiva- lent	Result
	No.	Weight			
A (Euglobulin, dialysis).....	1	185	4.5	4.0	+7'30"
	2	180	3.3	3.0	+3'40"
	3	192	2.2	2.0	Very severe shock
B (Euglobulin, CO_2).....	4	170	3.3	3.0	+5'30"
	5	195	2.2	2.0	Very severe shock
	6	190	2.7	2.5	Very severe shock
C (Pseudoglobulin + albumin)....	7	195	5.0	4.0	+4'20"
	8	190	2.5	2.0	+5'30"
	9	215	1.5	1.25	+2'30"

It will be observed that in this case a large amount of the total toxicity was recovered. The lethal dose of the anaphylatoxin employed being 0.25, the original 10 c.c of serum contained 10/0.25, or 40 lethal doses; 10/3.0, or 3.3 lethal doses, were recovered from A and B, respectively, totalling 6.6 for the euglobulin, while at least 10/1.25, or 8 m. l. d. were recovered from the water soluble portion of the serum. Unfortunately, not enough of solution C remained to complete the test. Neglecting this, between 14 and 15 lethal doses were recovered from the 40 originally present.

What is the cause of the small amount of the toxic principle recovered? The collodion sacs used were very thin, and it is possible that some of the toxin might be lost by diffusion through the sac. This point was tested by dialyzing against changes of distilled water. The dialysates were pooled and concentrated in a current of warm air to a small volume, but in no case were we able to demonstrate toxicity. Experiments outlined below under "Ultra-Filtration" will

bring confirmation to the idea that very little or none of the toxic principle passes a collodion sac made in the ordinary manner. Another possibility is that the toxic principle is quite unstable, and that in the presence of distilled water it may change to a harmless product. Strength is lent to this view by the fact that euglobulin undergoes marked changes when it remains for any time in contact with distilled water. It was thought advisable, therefore, to reduce as much as possible the time consumed in dialysis, endeavoring to obtain at the same time the greatest possible yield of euglobulin.

For this purpose the method of Heynsius²⁴ was employed. This investigator demonstrated that when serum was exactly neutralized previous to dialysis the flocking out of euglobulin occurred with considerably greater speed than was otherwise the case, but that the contents of the sac soon became alkaline again. Consequently, to insure neutralization at the end of the reaction it was necessary to add a considerable excess of acid at the start. By this method a large amount of euglobulin can be obtained in a very short time.

Rat anaphylatoxin was prepared in the usual manner and the minimal lethal dose was found to be 0.35 cc for a guinea-pig of 200 gm. weight. Ten cc were acidified with 1.5 cc of N/5 HCl, placed at once in a thin collodion sac and dialyzed against running distilled water. A heavy precipitate had separated in 30 minutes. To insure a maximal yield the dialysis was continued for 8 hours.

A. The precipitate was centrifugated out at 8,000 revolutions and dissolved in 20 cc (twice volume), of 0.85% NaCl solution. It has been mentioned before that the toxicity of anaphylatoxic euglobulin varies directly with the degree of the dispersion of its solutions. Consequently the euglobulin solution just made was tested for toxicity in the following manner.

1. The first tests were made with the double volume 0.85% NaCl solution A.
2. After test of 1 the balance of the solution (11 cc), were diluted to 22 cc (4 times volume), and tested.
3. The balance of 2, after the test injections, was diluted to 24 cc with 0.85% NaCl (6 times volume), and tested.
4. After the injection of the tests of 3, 1 cc of N/25 Na₂CO₃ was added to the balance of the solution. The solution, which through 1, 2 and 3 had been opalescent, became perfectly clear. It was tested at once.

The result of all the tests is recorded in Table 3.

The result of the experiment recorded in Table 3 is far better than the previous ones. By dilution and addition of alkali to form the so-called "salt alkali globulin," the m. l. d. of the euglobulin recovered by this method of dialysis was reduced to between 1.0 and 1.17 cc, a yield of at least 32% of the total original toxicity.

²⁴ Pflüger's Arch. f. Physiol., 1876, 12, p. 549.

That practically all of the water insoluble globulin was removed by this 8-hour acid-dialysis is shown by the fact that bubbling of CO_2 through the supernatant failed to bring down any further precipitate. Similar experiments have confirmed this method as the most effective one for recovering the euglobulin toxicity by dialysis.

TABLE 3
ACID DIALYSIS (HEYNSIUS) OF ANAPHYLATOXIN

Solution	Guinea-Pig		C C Intra- venously	Serum Equiva- lent	Result
	No.	Weight			
1 2 \times volume.....	1	180	2.0	1.0	Fair shock
	2	190	3.0	1.5	Severe shock
	3	178	4.0	2.0	+3'50"
2 4 \times volume.....	4	225	6.0	1.5	+3'10"
3 6 \times volume.....	5	182	6.0	1.0	Severe shock
4 6 \times volume + alkali.....	6	195	6.0	1.0	Near-kill
	7	180	7.0	1.17	+4'50"

It has been shown by Chick¹⁹ that by long continued dialysis at a slightly higher than ordinary temperature all of the globulin in serum can be made water insoluble. We attempted this experiment, thinking that in this way we might recover not only the toxic principle attached to the ordinarily present water-insoluble globulin, but that the portion which usually remained with the water-soluble fractions might precipitate as this changed to the insoluble form. The temperature used for this experiment was 45 C., a constant temperature being maintained by suspending the dialyzing sac in a Roux bath.

Eleven c c of an anaphylatoxin of an m. l. d. of 0.35 c c were placed in a thin collodion sac and dialyzed under the conditions just described.

A. After dialysis for 18 hours the resulting heavy precipitate was centrifuged out and dissolved in 21.5 c c of 0.85% NaCl + 0.5 c c of N/25 Na_2CO_3 (2 \times vol.).

B. The supernatant from A was bubbled with CO_2 for 10 minutes, the resulting gummy precipitate centrifuged out, and similarly dissolved in NaCl and alkali (2 \times vol.).

C. The supernatant from B was returned to the sac and dialyzed for an additional 17 hours and the small resulting precipitate dissolved in the same way as A and B.

D. The supernatant from C was bubbled with CO_2 . The precipitate was very scanty, so the solution was returned to the dialyzer, without centrifugation, for 18 hours additional dialysis. The resulting precipitate, which was still very scanty, was dissolved as in the case of A, B, and C.

E. Supernatant fluid remaining from the last dialysis was concentrated in a warm air current, tested for toxicity, and for presence of globulins by the additions of increasing amounts of $(\text{NH}_4)_2\text{SO}_4$.

The result of all the toxicity tests is recorded in Table 4.

TABLE 4
DIALYSIS OF ANAPHYLATOXIN AT 45 C., ATTEMPTED RECOVERY OF TOTAL GLOBULIN

Solution	Guinea-Pig		C C Intra- venously	Serum Equiv- alent	Result
	No.	Weight			
A (Euglobulin 18-hour dialysis)....	1	210	8.0	4.0	+3'30"
	2	195	5.0	2.5	+5'30"
	3	200	2.5	1.25	Moderate
	4	192	4.0	2.0	+5'30"
B (Euglobulin CO_2).....	5	192	8.0	4.0	Moderate
	6	200	10.0	5.0	Moderate
C (Euglobulin 35-hour dialysis)....	7	192	6.0	3.0	Nil
	8	200	10.0	5.0	Very slight
D (Euglobulin 53-hour dialysis)....	9	190	6.0	3.0	Nil
	10	200	10.0	5.0	Very slight
E (Supernatant, albumin).....	11	200	4.0	4.0	Nil
	12	205	6.0	6.0	Moderate

It will be noticed that only in the case of the globulin from the 12-hour dialysis is any appreciable amount of toxin recovered, and in this case only 5.5 out of a possible 31 m. l. d. It is probable that the high temperature of this experiment helped to destroy much of the already unstable toxic principle. It is worthy of note that tests made on solution E showed no precipitate with 44 and 47% saturation, a faint opalescence with 50%, and a marked precipitate with 57% saturation with $(\text{NH}_4)_2\text{SO}_4$.

Of the various methods of dialysis attempted, that in which the serum is first acidified is by long odds the method of choice. In the case of ordinary dialysis for 24 hours the yields are scanty, and at best most irregular. This is probably due, in the case of dialysis at ordinary temperatures, to the long time necessary to obtain an appreciable yield of euglobulin (for euglobulin precipitation was always incomplete at the end of 24 hours' dialysis), and in the case of that at 45 C. to the effect of the comparatively high temperature on the toxic principle.

FRACTIONATION OF ANAPHYLATOXIN BY DILUTION AND ACIDIFICATION

On account of the objections to the method of dialysis just described, it was thought wise to attempt the precipitation of the euglobulin by the more rapid method of dilution and acidification

The first experiment was performed by diluting anaphylatoxic serum with 9 volumes of distilled water and immediately afterward passing CO_2 through the diluted fluid for 15-30 minutes.

A. The anaphylatoxin was made in the usual manner, and on test its m. l. d. was found to 0.25 c c. Ten c c of this serum were diluted with 90 c c of distilled water and CO_2 passed through for 30 minutes. The resulting flocculent precipitate was centrifugated out at 6,000 revolutions and iced for 2 hours. Following this, it was dissolved in 20 c c of 0.85% NaCl solution.

1. This solution ($2 \times$ vol.) was tested for toxicity.

2. Solution 1 being very opalescent, its dispersion was increased by the addition of $\frac{1}{4}$ th volume of N/40 Na_2CO_3 . The solution became clear and was tested for toxicity.

B. The supernatant remaining after centrifugation of the euglobulin precipitate was cooled to 0 C. Its volume was 100 c c, and to it were added 100 c c of 0 C. neutral saturated $(\text{NH}_4)_2\text{SO}_4$. The heavy precipitate separating out was filtered on a Buchner funnel, redissolved in 10 c c of distilled water, and dialyzed in a collodion sac for 18 hours. A slight precipitate showed that the CO_2 had not removed all of the euglobulin. This was removed by centrifugation, and the supernatant, consisting of pseudoglobulin, was concentrated in a warm air current to 9.5 c c and brought to isotonicity by the addition of 0.5 c c of 17% NaCl. Solution B was then tested for toxicity.

C. The filtrate (albumin) from the precipitate B (pseudoglobulin), was saturated with crystals of $(\text{NH}_4)_2\text{SO}_4$, filtered on a Buchner funnel, and the precipitate dissolved in 10 c c of distilled water, and dialyzed for 18 hours. Even this fraction showed traces of euglobulin. These were centrifugated out at the completion of dialysis, the supernatant concentrated to 9.5 c c isotonized with 0.5 c c of 17% NaCl solution and tested.

The results of this experiment are recorded in Table 5.

TABLE 5
THE FRACTIONATION OF ANAPHYLATOXIN BY DILUTION AND CO_2

Solution	Guinea-Pig		C C Intra- venously	Serum Equiva- lent	Result
	No.	Weight			
A (CO_2 euglobulin) 1.....	1	200	2.0	1.0	Very severe shock +4'20"
	2	205	2.2	1.1	
	3	200	2.5	1.0	+3'
	4	195	2.1	0.8	Near-kill
	5	200	2.3	0.9	+3'40"
B (Pseudoglobulin).....	6	215	3.5	3.5	Nil
	7	200	6.5	6.5	Nil
C (Albumin).....	8	207	10.0	10.0	Nil

It will be seen by reference to Table 5 that all of the toxic principle recovered was found on the euglobulin precipitated by dilution and CO_2 . The traces of euglobulin flocking out on dialysis of the pseudoglobulin and albumin fractions were tested for toxicity and found to produce no effect. The number of lethal doses recovered on the euglobulin A_2 may be calculated to be $10/0.8$, or 12. The

original anaphylatoxin before dialysis contained 10/0.25, or 40 lethal doses. Consequently, 30% of the original toxicity was recovered on the water-insoluble globulin. No toxin could be demonstrated in this instance on the pseudoglobulin or albumin fraction, as reference to B and C (Table 5) will show. It is by no means certain, however, that some of the toxicity was not present in the water-soluble portions originally, since we have found that the toxic principle is quite unstable in the presence of large amounts of $(\text{NH}_4)_2\text{SO}_4$; this is likewise the case when high concentrations of MgSO_4 , $\text{MgNa}_2(\text{SO}_4)_2$, and NaCl are used in fractionation.

Anaphylatoxin from this same batch was used for a test with dilution and acetic acid. The material was treated exactly as in the foregoing experiment except that 1.6 c c of N/10 acetic acid, instead of CO_2 , were added to 100 c c of the dilute serum. In this case the salt alkali euglobulin was fatal to guinea-pigs in a dose of 1 c c serum equivalent. The results differ from those of the preceding experiment, however, in that some of the toxic principle could be demonstrated on the water-soluble globulin (equivalent of B, Table 5). The lethal serum-equivalent of this fraction was 3.5 c c. The total number of lethal doses recovered in this experiment was 12.8 on the euglobulin, and 2.8 on the pseudoglobulin. The total toxicity recovered was 33% of the original.

It is not to be inferred that the recoverable toxic principle is always confined to the euglobulin when anaphylatoxin is fractionated by the dilution and CO_2 method. In one instance where a sample of anaphylatoxin with a m. l. d. of 0.25 was diluted with 9 volumes of distilled water and CO_2 passed through for 30 minutes, 1 c c of euglobulin dissolved in twice the original volume of alkaline NaCl solution was fatal to a guinea-pig of 200 gm. weight. The supernatant was concentrated to original volume, made isotonic, and found to be fatal in 0.9 c c dose. Bearing in mind that the original 10 c c of anaphylatoxin contained 10/0.25 or 40 m. l. d., and that 10/1.0 were isolated on the euglobulin and 10/0.9 on the pseudoglobulin-albumin fraction, a total of 21, or more than 50% were recovered in this instance. The water soluble proteins were not in this instance fractionated with $(\text{NH}_4)_2\text{SO}_4$, a fact which strengthens the hypothesis as to the cause of loss of toxicity in the preceding experiment.

It is a curious fact that the yields of anaphylatoxin recovered after fractionation of anaphylatoxin prepared by the less efficient direct

gel method are relatively greater than when the sol-gel method is used. This has been found to be the case with both rat and guinea-pig serum anaphylatoxin, and is well illustrated by the following experiment.

Six c.c. of fresh rat serum were shaken up with 1.25 c.c. of 0 C. agar gel and the mixture incubated for 30 minutes. The agar was then removed by centrifugation and the lethal dose, on test, was found to be 0.75 c.c. for guinea-pigs of 200 gm. weight.

A. Four and one-half c.c. of this anaphylatoxin were diluted with 36 c.c. of distilled water and CO_2 was passed through this dilute serum for 20 minutes. The euglobulin precipitate was removed by centrifugation and dissolved in 2 volumes (9 c.c.) of 0.85% NaCl solution. The solution, which was opalescent, was cleared with 2 c.c. of N/40 Na_2CO_3 , and tested for toxicity on guinea-pigs.

B. The supernatant, consisting of pseudoglobulin and albumin, was concentrated to original volume, made isotonic, and tested as in the case of A.

The results of this experiment are given in Table 6.

TABLE 6
FRACTIONATION OF DIRECT GEL RAT ANAPHYLATOXIN

Solution	Guinea-Pig		C C Intra- venously	Serum EQUIVA- lent	Result
	No.	Weight			
A.....	1	197	3.7	1.5	Light shock +6'30"
	2	190	4.9	2.0	
B.....	3	205	3.0	1.5	Severe shock +3'50"
	4	212	4.0	2.0	

Calculation shows that a total of approximately 75% of the original toxicity was recovered in this instance—37.5% on the CO_2 euglobulin and 37.5% on the water-soluble proteins.

Similar experiments were made with guinea-pig serum. Here the same phenomenon held true, that is, the anaphylatoxin of comparatively low potency prepared by the direct gel method yielded far more of the toxic principle on fractionation than did more highly toxic serum prepared by the sol-gel method. The precipitation of the euglobulin by the dilution and CO_2 method gives the greatest yield of toxin in the case of guinea-pig serum. In several instances 75% of the total original toxicity were recovered on the water-insoluble globulin.

Repeated attempts were made to fractionate anaphylatoxins directly with $(\text{NH}_4)_2\text{SO}_4$, but the results were very disappointing. It is well known that single precipitations yield fractions by no means pure. Haslam has shown that at times 17 reprecipitations are required to obtain pure products. We attempted fractional precipitation of

anaphylatoxic serum with $(\text{NH}_4)_2\text{SO}_4$ and found that while a large amount of the toxic principle can be recovered on the first precipitates, purification results in a rapid disappearance of all of the toxic principle. The attempts were consequently abandoned.

Hardy and Gardiner²⁵ have described a method of isolating the total serum protein, freed from all but a minimal amount of other serum constituents, and with the various physiologically active principles intact. The method consists essentially in the precipitation of the serum in the cold with several volumes of absolute alcohol or acetone, and the subsequent careful extraction of the precipitate with ether. The resulting protein powder is easily soluble in distilled water, and by reason of the removal of a large amount of lipid substance, is subject to more clear cut fractionation than the whole serum.

PRECIPITATION OF ANAPHYLATOXIC PROTEIN BY THE METHOD OF HARDY AND GARDINER

The first experiment made was a determination of the amount of anaphylatoxic principle recoverable by this method.

Rat anaphylatoxin was made by the usual sol-gel method and found to be fatal to guinea-pigs of 200 gm. weight in dose of 0.25 c.c. Twenty-four c.c of this serum were cooled to 0 C. and thrown into 120 c.c of -8°C . absolute alcohol. The resulting heavy precipitate was at once filtered off by suction on a previously chilled Buchner funnel and washed with cold anhydrous ether. Following this the precipitate was transferred to a beaker and extracted for 30 minutes with boiling ether. The resulting product, which was considerably caked, was comminuted with a spatula and transferred to a desiccator, where it was kept in vacuo over H_2SO_4 till thoroughly dry.

On the following day the powder was dissolved in original volume (24 c.c) of distilled water, forming a perfectly clear, golden yellow solution. This solution, tested by intravenous injection into guinea-pigs of 200 gm. weight gave a very severe shock in 0.3 c.c and was lethal in 0.35 c.c.

As is the case with most principles whose activity depends to a great extent on their colloidal nature, this protein powder forms an excellent means of preserving the anaphylatoxin for great lengths of time. Acetone proved to be even more effective than alcohol, repeated trials with the former reagent giving products which retained the toxic principle quantitatively. It is of the highest importance to use cold reagents in performing this experiment. The serum should be cooled to 0 C. and the acetone to -8 to -14°C . It is advisable, moreover, to provide a jacket for the Buchner funnel in order that this may be kept at as low a temperature as possible.

²⁵ Proceedings Physiol. Soc., reprinted in Jour. Physiol., 1910, 40, p. 68.

Having determined that the anaphylatoxic principle is very well preserved by this method the next step was to discover whether solutions of this toxic protein would yield highly toxic fractions.

Dry rat anaphylatoxin protein equivalent to 5.5 cc of serum was dissolved in 11 cc (double volume) of distilled water. On test the m. l. d. of this protein solution was found to be 0.35 cc. This solution was diluted with 10 volumes of distilled water and CO_2 passed through it for 30 minutes. The resulting precipitate was centrifugated out at 8,000 r. p. m.

A. 1. The euglobulin precipitate was dissolved in 22 cc of 0.85% NaCl ($4 \times$ volume) and tested.

2. Solution 1 being very opalescent, the dilution was increased to 8 volume and tested.

3. A slight opalescence persisted in solution 2—it was accordingly treated with 2 cc of N/40 Na_2CO_3 and as a result immediately became water clear. This was tested as before.

B. The supernatant from A was cooled to 0 C. and was thrown into an equal volume of 0 C. neutral saturated $(\text{NH}_4)_2\text{SO}_4$ solution. The precipitate (pseudoglobulin) was centrifugated out, dissolved in 10 cc of distilled water and dialyzed in a thin collodion sac for 24 hours. A small precipitate consisting of a trace of euglobulin resulted. This was centrifugated out, dissolved in NaCl solution and tested. The supernatant was concentrated to original volume, made isotonic and tested.

C. The supernatant from the precipitate B, coming down on half saturation with $(\text{NH}_4)_2\text{SO}_4$ was concentrated in a warm air current till crystals of the salt appeared, showing full saturation. The precipitate was filtered off on a Buchner funnel, redissolved, reprecipitated, and finally redissolved in distilled water and dialyzed for 24 hours. No precipitate resulted. The dialysee was concentrated to original volume and isotonized, following which it was tested for toxicity.

The result of these tests is recorded in Table 7.

TABLE 7
THE FRACTIONATION BY CO_2 AND $(\text{NH}_4)_2\text{SO}_4$ OF HARDY PROTEIN FROM RAT ANAPHYLATOXIN

Solution	Guinea-Pig		C C Intra- venously	Serum Equiva- lent	Result
	No.	Weight			
A (Euglobulin) 1.....	1	175	2.8	0.7	Severe shock
	2	180	3.2	0.8	Severe shock
	3	183	3.6	0.9	+4'10"
2.....	4	170	6.4	0.8	+3'30"
	5	200	5.6	0.7	Very severe shock
3.....	6	180	6.0	0.625	Near-kill
B (Pseudoglobulin).....	7	210	3.5	3.5	Nil
C (Albumin).....	8	205	5.5	5.5	Nil

The result is an excellent one and surpasses by far, in regard to yield of toxin, the ones obtained by similar fractionation applied to raw serum. The original protein solution contained 5.5/0.35, or 15 m. l. d.—5.5/0.625, or 8 m. l. d., were recovered on the euglobulin

fraction, and to this must be added at least 1 m. l. d. obtained from the trace of euglobulin coming down during the dialysis of B (pseudoglobulin), for this, when dissolved in 1 c.c. of NaCl solution and injected, killed a guinea-pig of 200 gm. weight. This total of 9 m. l. d. out of a possible 15 recovered gives a yield of nearly 60% on the euglobulin fraction.

The high toxic yields after fractionation were not confined to the method of dilution and CO_2 , but were surprisingly good when the Hardy protein was half saturated with $(\text{NH}_4)_2\text{SO}_4$. This method, when applied to anaphylatoxic whole serum gave most inconstant results. On the other hand, when anaphylatoxic protein isolated by the Hardy method was treated in this manner and the resulting fractions dialyzed, the resulting euglobulin precipitate, when dissolved and injected, proved to possess 50% of the total original toxicity. The pseudoglobulin and albumin fractions, injected in large doses, proved to be nontoxic.

Besides the high yields of toxic principle recovered by fractionation of the anaphylatoxic Hardy protein, another result of the greatest importance should not be overlooked. That is the relatively specific manner in which the toxin is associated with the euglobulin fraction. In one case only, where the method used was the just described half saturation with $(\text{NH}_4)_2\text{SO}_4$ followed by dialysis, did we succeed in recovering any toxicity on the water-soluble globulin. In this instance out of a total of 9.2 m. l. d. recovered, 45 out of a total of 68% were demonstrated on the euglobulin. The remainder was about equally distributed between the pseudoglobulin and albumin fractions.

A point in this experiment well worthy of note is the character of the shocks and necropsy pictures in the guinea-pigs dying from the pseudoglobulin and albumin injections. In both instances the shock was of the subacute type described by one of us in a preceding paper.²⁶ The deaths, instead of taking place in 2-4 minutes, as is usually the case, occurred in 7 minutes 50 seconds in the case of the pseudoglobulin, and in 51 minutes in the case of the albumin. A bloody foam exuded from the nostrils during the shock, and a marked pulmonary edema was demonstrable at necropsy. This picture is characteristic of the shock produced by the injection of primarily toxic normal and immune rabbit serums, and as we shall demonstrate below, the toxin in these instances is strictly associated with the water-soluble proteins of such serums.

* DeKruif: *Jour. Infect. Dis.*, 1917, 20, p. 717.

It might be well at this point to sum up the results obtained by the various methods of fractionation so far described. Dialysis of the whole serum anaphylatoxin yields at best inconstant results. The unreliability of this method appears to be due to the time consumed in the dialysis and the incompleteness of the euglobulin precipitation. The euglobulin (water-insoluble) fraction which carries the greater part of the toxicity is known to be sensitive to changes produced by too long contact with distilled water, and it is this fact that very probably is the cause of the failure to recover large amounts of toxin by this method. When the speed of dialysis flocculation of the euglobulin is increased by the addition of acid to the serum the results obtained are much better, as much as 32% of the total original toxicity being recoverable on the euglobulin fraction.

Dilution and acidification of the whole serum anaphylatoxin has the advantage of speed over even the acid method of dialysis just described. The toxin yields are quite constant, although they are not much greater in amount than those obtained by acid dialysis. In the majority of instances the greater part of the toxic principle recovered is associated with the water-insoluble globulin fraction, but in some cases a partition between the water soluble and insoluble fractions occurs. The method of preparation of the anaphylatoxin seems to affect the yield of this substance obtained on fractionation. The less efficient method of the addition of agar gel to serum giving better relative results than the sol-gel method.

When the serum proteins from anaphylatoxin are precipitated by alcohol or by acetone in the cold and extracted with dry ether, the anaphylatoxic principle can be recovered quantitatively on redissolving these proteins. The quantitative recovery is most regularly obtained when acetone is used as the precipitant. It is highly desirable to have the reagents and the apparatus used thoroughly chilled. Such protein solutions yield much higher proportions of the toxic principle on fractionation than do whole serum anaphylatoxins. What is more, the toxic principle recovered appears almost invariably and exclusively on the euglobulin.

THE ULTRA-FILTRATION OF ANAPHYLATOXIN

The methods of fractionation employed in the first part of this paper have given valuable evidence as to the location of the anaphylatoxic principle in serum. It would seem, however, in view of the delicately balanced equilibrium of the serum colloids, that the frac-

tionation procedure might introduce a too violently disturbing factor, which might be the cause of our invariable failure to recover the toxic principle quantitatively on one fraction.

If some purely physical method could be found to effect this separation of the poison from the bulk of remaining serum constituents, greater progress might be made in these studies. The first method attempted was that of ultra-filtration, devised by H. Bechhold.²⁷ This author is able to separate colloids of different grades of dispersion by means of membranes constructed by impregnating filter papers with gels of one kind or another. That most frequently used is an acetic acid celloidin. By varying the concentration of celloidin in the acetic acid, membranes of varying permeability are secured.

This method, after thorough trial, was discarded as unsuitable. The greatest difficulty was met with in securing any regularity in membrane permeability, and what is more, the individual membranes varied greatly in permeability at different parts of their surfaces.

Our next trials were made with the method of Schoep²⁸ and met with unexpectedly good results. The membrane used in this case is prepared from a solution of celloidin in alcohol-ether, the permeability of the celloidin being modified by the introduction of castor oil and glycerol. This filter is most conveniently used in the form of a dialyzing sac. The degree of permeability can be quite accurately adjusted. We will outline a description of the technic employed.

Two solutions, A and B, are prepared as follows:

Solution A

Schering's celloidin	4 gm.
Absolute alcohol	15 c c.
Dry ether	85 c c.

When dissolved add

Castor oil	4 c c.
Glycerol	4 c c.
Absolute alcohol until clear, about.....	15 c c.

Solution B

Schering's celloidin	4 gm.
Absolute alcohol	50 c c.
Dry ether	50 c c.

By mixing solutions A and B in different proportions, membranes of different permeability may be prepared. The more permeable the membrane desired, the more of A is used, and vice versa. Different batches of the solutions made up at different times, do not necessarily yield membranes of exactly

²⁷ Ztschr. f. physik. Chem., 1907, 60, p. 237.

²⁸ Koll. Ztschr., 1911, 8, p. 80.

like permeability, so that the proper ratio of A to B must be determined for each batch of material made up.

Method of Making the Sacs.—The Novy-Gorsline tubes were used in preparing the sacs, the general method of preparation of which is identical with that of the ordinary celloidin dialyzing bag. A little celloidin is placed over the hole at the end of the tube and allowed to dry. The tube is then immersed in a cylinder containing the desired mixture of A and B, rotated a few times, and after gently shaking off the excess of celloidin is withdrawn from the cylinder and rotated rapidly in a horizontal position for 15-30 seconds. A second coat is now applied in the same way and if necessary a third, rotation to secure even distribution being practiced each time. The celloidin is allowed to dry on the tube until it becomes opaque and whitish. The tube is then filled with water, and after thrusting it into a large beaker of water, air pressure is applied gently to the mouth, with the result that the water within the tube passes out through the hole in the bottom and between the tube wall and the sac. The sac is thus easily disengaged from the tube.

The sac is now carefully attached to a cut-off test tube, the seal being made air-tight and tested for leakage. The apparatus is then lowered into a cylinder, and immersed in distilled water. The fluid to be filtered is introduced, the top of the cut-off test tube is closed off with a single hole rubber stopper through which the pressure line is introduced. A mercury manometer is placed in the pressure line and the filtration allowed to proceed at a pressure of not more than 100 mm. Greater pressures than this endanger the sac, the walls of which are delicate and easily ruptured.

The process of filtration usually consumes from 30 minutes to 2 hours, depending on the amount of fluid filtered and the permeability of the membrane. The volume of the fluid introduced is usually decreased from 4-6 times. The same sac can be used for more than one filtration if the precaution is taken to force a large quantity of distilled water through the bag at once after the completion of the operation.

The fluid remaining inside the bag after filtration we will designate as supernatant, that found on the outside will be called the filtrate. Since much of the electrolyte is filtered off during the operation, the supernatants were always brought to tonicity before injection, and diluted to the original serum volume. The supernatants were concentrated to a workable volume in a warm air current.

It will be apparent that this method is only of value if accurate estimation of the protein contents of the supernatant and of the filtrate is made. Only in this way can an estimation of the size of the aggregates carrying the toxic principle be formed. The refractometric method of Robertson is considered the most suitable for estimation of the protein content of serums. Unfortunately, one of these instruments was not available, so we were compelled to resort to the method of acidification, addition of salt, boiling and weighing. Filter papers dried to a constant weight were used, and in all the estimations the drying of the protein precipitates on the filters was most carefully conducted.

In the majority of instances the total protein of the supernatant and filtrate, respectively, was estimated. In cases where the euglobulin, pseudoglobulin and albumin were separately determined, the euglobulin was removed by dilution and CO_2 and redissolved. The supernatant from this precipitate was then half saturated with $(\text{NH}_4)_2\text{SO}_4$, and the thus separated pseudoglobulin and albumin fraction reprecipitated and redissolved. Finally, all three solutions were made acid to litmus with N/10 acetic, enough $(\text{NH}_4)_2\text{SO}_4$ added to make about a 10% solution, boiled for 30 minutes, and the resulting precipitates were filtered and washed with hot water until free from sulphate, dried and weighed.

The experiments with the Schoep method of ultra-filtration were conducted with both rat and guinea-pig anaphylatoxin. The size of the colloidal aggregates with which the toxic principle is associated is smaller in the case of rat serum than in that of the guinea-pig. As a result the membranes holding back the former were composed of proportionately more of solution B.

Eleven c.c. of rat anaphylatoxin were made by the sol-gel method and on test the m. l. d. was found to be 0.5 c.c. This serum was diluted to 55 c.c. with distilled water and placed in a 50A-50B sac. The sac was surrounded by a small amount of distilled water and the filtration conducted at a pressure of 80 mm. of mercury, for 1 hour and 45 minutes, until the fluid in the sac was reduced to 15 c.c. Five c.c. of physiological salt solution were used to rinse out the sac; this was added to the supernatant, which was then brought to double the original volume by the addition of 2 c.c. of 17% NaCl solution. The result of the toxicity tests and protein estimation is given under A, Table 8.

The filtrate B was reduced to double volume in a warm air current. This was also tested for toxicity and a determination of protein was made.

TABLE 8
SHOWING RESULT OF TOXICITY TESTS AND PROTEIN ESTIMATION

Solution	Guinea-Pig		C C Intra- venously	Serum Equiva- lent	Result
	No.	Weight			
A (Supernatant).....	1	200	2.0	1.0	+4'30"
	2	180	1.5	0.75	+2'55"
	3	180	1.0	0.5	Near-kill
B (Filtrate).....	4	200	10.0	5.0	Nil

Of the total protein recovered, 48.3% was recovered from the supernatant A and 51.7% from the filtrate B. At the same time observation of Table 8 will show that the toxin was retained practically intact within the sac. This experiment would indicate that

the toxic principle must be part of some large colloidal aggregate that passes the ultra-filter with great difficulty, if at all, even when the porosity is great enough to pass a large portion of the serum protein.

It was thought desirable to filter anaphylatoxin repeatedly, diluting it each time after a portion of the fluid had been forced through. This experiment, the result of which proved to be interesting, was conducted as follows.

Fifteen cc of rat anaphylatoxin the m. l. d. of which was 0.35 cc, were diluted with 55 cc of distilled water and placed in a 50A-50B sac.

Filtration 1. One hundred mm. of pressure, 2 hours and 30 minutes till volume of supernatant was 30 cc. Sac then washed and 50 cc of distilled water forced through; 0.7 cc removed for test.

Filtration 2. Supernatant 1 diluted with distilled water to 50 cc, 100 mm. of pressure, 2 hours till volume was 29.7 cc. Sac washed as before. Portion removed for toxicity test.

Filtration 3. Supernatant 2 diluted to 50 cc with distilled water, 100 mm. of pressure, 1 hour and 45 minutes till volume was 26.7 cc. Tested for toxicity and protein content determined.

The filtrate from filtration 1 was concentrated to original volume and tested for toxicity and the protein content determined.

The filtrates from filtrations 2 and 3 combined, concentrated to original volume, tested for toxicity and protein content determined.

The result of the foregoing experiment is recorded in Table 9.

TABLE 9
REPEATED ULTRA-FILTRATION OF A GIVEN SAMPLE OF ANAPHYLATOXIN

Solution	Guinea-Pig		C C Intra- venously	Serum EQUIVA- lent	Result	Protein Content, Per Cent.
	No.	Weight				
Filtration 1						
(Supernatant).....	1	195	0.7	0.35	+3'50"	50
(Filtrate).....	2	210	5.0	5.0	Nil	
Filtration 2						
(Supernatant).....	3	190	0.7	0.35	Heavy shock	
	4	200	0.8	0.4	Very severe	
	5	210	0.9	0.45	Very severe	
	6	200	1.0	0.5	+5'40"	
Filtration 3						
(Supernatant).....	7	200	1.25	0.625	Moderate	14
	8	210	1.6	0.8	Moderate	
	9	200	2.0	1.0	+5'40"	
(Filtrate 2 + 3).....	10	180	2.0	2.0	+4'10"	36
	11	190	1.0	1.0	Nil	
	12	175	1.5	1.5	Nil	

It will be seen from Table 9 that though half the total recovered protein was found in the first filtrate, all of the original toxicity could be recovered in the supernatant. When the supernatant was diluted a second time, filtered, and tested, there was some drop in toxicity.

the lethal doses being 0.5 instead of 0.35 c c as in the original serum and in supernatant 1. The second and third filtrates were pooled, so that the toxicity demonstrated and the quantity of protein determined must be the sum of filtrations 2 and 3. The toxicity test of these pooled filtrates shows that some of the toxic principle has passed the membrane (Guinea-pig 10, Table 9). On the other hand, the amount of toxic principle to be found here is very small, —7.5 m. l. d. This will be seen to be true, since reference to Guinea-pig 9, Table 9, shows that 13.3 m. l. d. were kept back in the third supernatant, even though this contained only 14% of the total recoverable protein.

Similar tests of multiple filtration of a given sample of anaphylatoxin confirm this finding. It is probable that repeated dilution of the toxic serum increases the dispersion of the large toxic aggregates sufficiently to allow a small amount to pass the membrane. This experiment strikingly confirms the idea of the association of the toxic principle with large colloidal complexes.

Guinea-pig anaphylatoxin was subjected to this ultra-filtration with similar results. In several instances the total toxicity was retained within the sac, while the filtrate was shown to contain at least 50% of the recoverable protein.

We have used the term "recoverable" protein in the foregoing experiments because when determinations of the protein content of unfiltered anaphylatoxin were run, values considerably in excess of the sum of the protein of the supernatant and filtrate were obtained. It was at once presumed that this discrepancy was due to adsorption of a portion of the protein in the membrane during the process of filtration. Should this be true, the results obtained would be even more striking, especially in cases where the total toxicity was recovered in the sac content after completion of filtration.

This deduction, when put to the test was found to be correct. An experiment of this kind merits detailed description because it shows the method devised for recovery of the protein held in the membrane.

Twenty c c of guinea-pig anaphylatoxin made by the sol-gel method and tested for its m. l. d. was found to be lethal in dose of 4 c c. It was diluted to 100 c c with distilled water and placed in a 5A-4B ultra-filter sac. It was filtered under 100 mm. pressure till the supernatant volume was 15 c c. One c c of 17% NaCl solution was used to make the liquid isotonic, the inside of the sac was washed with 0.85% NaCl, and this wash-water was added to the supernatant, bringing its volume back to that of the original serum (20 c c). The supernatant was now tested for toxicity and the remainder used for determination of the protein content.

The filtrate was concentrated to original volume in a warm air current, the toxicity tested and the protein content determined.

The sac used in this experiment was dried in air for 1 hour and in a desiccator for 2 hours, cut up into very small pieces and covered with alcohol and ether. This was allowed to stand overnight and in the morning, after alcohol and ether added the evening before had been decanted, was shaken up with fresh alcohol and ether and allowed to stand for 15 hours. For a control, another sac was prepared and treated in the same manner, but without being used for filtration. It completely dissolved in alcohol and ether in a few hours. The celloidin having then completely dissolved, the fluid was filtered off on a previously dried and weighed filter paper and the amount of sac protein determined by weighing.

The amount of protein in the unfiltered anaphylatoxin of the same pool was determined. The results of this experiment are recorded in Table 10.

TABLE 10
RETENTION OF SERUM PROTEIN IN ULTRA-FILTER SAC

	Amount of Protein in Gm.	Per Cent. of Protein	Toxicity (Lethal Dose)	Per Cent. of Toxicity
Supernatant.....	0.130	31	4.0	100
Filtrate.....	0.192	46	(No effect with 8 c c)	0
Sac.....	0.097	23		
Total.....	0.419	100		
Control (unfiltered anaphylatoxin)	0.413		4.0	

While the toxicity of the serum is completely recovered in the supernatant, only 31% of the total protein is to be found in this portion. The amount of the protein adsorbed in the membrane has varied in our experiments from 18-25% in the case of guinea-pig anaphylatoxin. The protein of rat anaphylatoxin seems to be retained in less amount, this varying from 7-20%. It will be apparent that the results given previous to this experiment would have been more striking, since the addition of the sac protein to that not found associated with the toxic principle would markedly increase the amount of toxin per unit quantity of protein recovered.

Of numerous experiments made with ultra-filtration the best result obtained has been the filtering off of 70% of the total protein with a total retention of toxicity within the sac. A fact worthy of note is that tests have shown the filtrates in cases where the toxicity has failed to pass the membrane to be free of water-insoluble globulin, that is, no precipitate was obtained when the filtrate was diluted and CO₂ bubbled through it for 30 minutes. On the other hand, the water-soluble (pseudo) globulin passes the membrane almost, though not quite, as freely as does the albumin. It would appear from this, then,

that while the euglobulin is not necessarily chemically different from the pseudoglobulin, its physical aggregates may be larger, possibly because of its combination with serum lipoid as suggested by Chick. It is unfortunate that we did not test the ability of normal serum euglobulin to pass these membranes. While the result might not be different, it is just possible that an actual aggregation may be a part of the process of anaphylatoxin production. Bordet,²⁹ indeed, observed that a heavy precipitate occurs when agar is added to unheated normal serum.

We have extracted many samples of the agar sediment centrifuged out after anaphylatoxin production and have not only succeeded in recovering considerable of the toxic principle, but by fractionation with $(\text{NH}_4)_2\text{SO}_4$ and dialysis have found the toxin to be associated with the euglobulin. It is possible then, that the whole explanation of the toxin production may lie in an aggregation of the globulin portion of serum under the influence of the colloid introduced. Plausibility is lent to this view by the peculiar phenomenon of distilled water toxification of normal and immune rat serums first observed by one of us in collaboration with Professor Novy.³⁰ It is well known that simple dilution produces spontaneous precipitation of the water-insoluble serum globulin. The temptation to correlate these two phenomena with those described in the preceding paragraphs is great, especially when one takes into account certain observations made in the course of this work.

We present the following observations tentatively because the phenomena observed have occurred irregularly and have so far been difficult to duplicate. This is perhaps due to the fact that we have not had time to determine all the factors which must be kept constant to bring about invariably the results we have observed a few times.

In an early paragraph it will be recalled that we made control injections of the euglobulin from normal rat serum and were able to inject 10 c.c serum equivalent of this protein without any harmful effect resulting. Since the toxic principle of anaphylatoxin is found to be associated closely with the euglobulin fraction, we attempted to make rat serum toxic by the agar sol-gel method after the euglobulin had been removed by dilution and acidification or by dialysis. It was found that the simple removal of this water insoluble globulin, which is a rather small constituent of the total rat serum protein, made a

²⁹ Compt. rend. Soc. de biol., 1913, 74, p. 1213.

³⁰ Novy and DeKruif: Jour. Infect. Dis., 1917, 20, p. 776.

profound change in the toxification reaction. The serum which before had been toxifiable in dose of 0.25 c c now could be made toxic only in very large doses (5-7 c c). In fact, this toxicity can easily have been a primary one.

Rat serum, then, loses its ability to become anaphylatoxic after removal of the water-insoluble globulin. On the other hand, when the water-insoluble globulin, freshly removed from serum, was mixed with agar and treated by the sol-gel method it apparently changed from a harmless state to one typically anaphylatoxic. It would be well to present the experiment in detail.

Nineteen c c of fresh normal rat serum were cooled to 0 C. and half saturated with cold neutral $(\text{NH}_4)_2\text{SO}_4$. The precipitate was centrifugated off and redissolved in 15 c c of distilled water and dialyzed for 24 hours. The heavy euglobulin precipitate was taken up in 19 c c (original volume) of 0.85% NaCl solution. The resulting solution was very opalescent and was therefore diluted to double volume by the addition of 19 c c more of normal salt. Twelve c c of this solution were set aside for control and the remainder treated as follows:

Twenty-three c c of euglobulin solution were warmed to 38 C. and mixed with 2 c c of 0.5% agar sol. The mixture was shaken, iced for 30 minutes, following which it was placed in the incubator for 30 minutes at 38 C. A flocculent precipitate similar to that forming when whole serum is toxified was visible at the end of this time. The agar was centrifugated out at 3,000 r. p. m. and the supernatant was tested for toxicity by intravenous injection into guinea-pigs of 200 gm. weight.

The result of this experiment is given in Table 11.

TABLE 11
THE TOXIFICATION OF RAT SERUM EUGLOBULIN BY AGAR

Solution	Guinea-Pig		C C Intra- venously	Serum EQUIVA- lent	Result
	No.	Weight			
Control (untreated globulin).....	1	180	11.0	5.5	Nil
Euglobulin + agar.....	2	175	7.0	3.5	+2'50"
	3	180	4.0	2.0	Moderate
	4	185	6.0	3.0	Severe

The picture of the shock and the findings at necropsy were perfectly typical of that produced by whole serum anaphylatoxin. Several attempts to duplicate this experiment resulted in failure, while several others for no explainable reason succeeded as well as the one described above. On the whole, it was found better to remove the euglobulin from fresh rat serum by dilution and CO_2 than by the method just described, for the reason that this product will undergo deleterious changes by long contact with distilled water.

These observations are of great interest because they immensely simplify the question of the mechanism of anaphylatoxin production. Instead of working with such an infinitely complex mixture of colloids as the whole serum, one is now able to study the euglobulin alone. This is far simpler than the former method, even taking into consideration the fact that the product used was not pure euglobulin.

This experiment awaits elaboration and careful control and we hope at some future time to concern ourselves exclusively with it. In the meanwhile the findings presented here support strongly the tentative hypothesis that serum toxification by foreign colloids is intimately related to some change, physical or chemical, in the water-insoluble globulin of the serum.

THE FRACTIONATION OF PRIMARILY TOXIC NORMAL AND IMMUNE SERA

This subject will only be touched on in a few words and is to be taken as a preliminary report of an investigation which will be elaborated in a later paper. It is well known that the normal serums of numerous species of animals are toxic for guinea-pigs. In the case of normal rat, rabbit, sheep and human serum we have invariably failed to find any of the primarily toxic principle associated with the water-insoluble globulin. On the contrary, in the case of sheep and rabbit serum more than 50% of this toxicity can be isolated on the pseudoglobulin fraction. The methods used in this investigation are entirely similar to those fractionations described in the first part of this paper.

It is a curious though well known fact that the serum of rabbits immunized with sheep red blood cells becomes extremely toxic for guinea-pigs. It was our good fortune, during the immunization of some rabbits with these cells, to obtain a sample of serum from a rabbit which had become cachectic under the injections. It had previously been noted by Jonesco Mihaiesti³¹ that serum of rabbits becoming cachectic under immunization is extremely toxic. The sample obtained by us confirmed this statement, since the serum was fatal to guinea-pigs on intravenous injection in dose of 0.05 c c. This toxicity decreased to 0.1 c c after the serum had been kept for a few days. A sample having this toxicity was precipitated with cold alcohol and extracted with ether by the method of Hardy and Gardiner already

³¹ *Compt. rend. Soc. de biol.*, 1913, 74, p. 1414.

described. The toxicity was recovered quantitatively when the resulting protein powder was redissolved.

Upon half saturation of this toxic protein solution with $(\text{NH}_4)_2\text{SO}_4$ and dialysis of the redissolved total globulin precipitate, the toxicity was found to be concentrated almost entirely on the pseudoglobulin fraction—0.1 c c of the protein solution was fatal to guinea-pigs, while 0.2 c c serum equivalent of the pseudoglobulin fraction produced the same effect. While the water-soluble globulin thus carried at least 50% of the original toxicity the euglobulin was found to possess only 3% and the albumin fraction none at all. This is in striking contrast to the results obtained with rat and guinea-pig anaphylatoxin, in which cases, as we have seen, the poison is almost entirely concentrated on the euglobulin fraction.

SUMMARY AND CONCLUSIONS

The toxic principle of rat and guinea-pig anaphylatoxins can be recovered on the globulin fractions of these serums.

Various methods of isolating these fractions have been studied. In the case of dialysis, very little of the toxic principle is recovered unless the speed and efficiency of this procedure is increased by the preliminary neutralization of the serum. Better results are obtained when the water-insoluble globulin is removed by dilution and CO_2 or dilution and acetic acid. In all of these cases the toxic principle appears on the water-insoluble rather than on the water-soluble fraction of the globulin, although in some instances partition between these fractions is found to occur. This seems to be due to the fact that the poison associated with the water-soluble fraction is much more unstable in the presence of high concentrations of $(\text{NH}_4)_2\text{SO}_4$ and other salts than is that associated with the water-insoluble fraction.

When anaphylatoxic serums are treated by the method of Hardy and Gardiner for the removal and purification of serum proteins, the toxic principle is quantitatively recoverable on the serum proteins. Such protein solutions, when fractionated, yield far more constant, more specific, and better quantitative results than those obtained by fractionating the whole serum. The toxic principle recovered from the fractionation of these protein solutions is found to be almost invariably and exclusively concentrated on the euglobulin fraction.

Anaphylatoxic serums have been further studied by the method of ultra-filtration. By the use of Schoep's methods most instructive

results have been obtained. The toxic principle has been shown to exist in extremely large colloidal aggregates which are retained even after as much as 70% of the total serum protein has passed the membrane. Quantitative determinations would indicate that very little or no water-insoluble globulin passes the filter, even when from 50% of the total protein has been recovered in the filtrate.

The foregoing results are important in that they furnish an excellent and simple means of concentrating the toxic principle, furnish material for a tentative hypothesis of the nature of the toxin, and effectively exclude the notion that protein degradation products are concerned in this toxicity. (The toxic principle of Witte peptone passes easily membranes which hold back all of the anaphylatoxin.)

It appears that euglobulin solutions freshly prepared from rat serum are toxifiable by the addition of agar. The results are inconstant, but have been reduplicated sufficiently to warrant their mention in this place.

A preliminary report of the fractionation of primarily toxic normal and immune serums has been made. Such toxins are not found on the euglobulin fraction as in the case of anaphylatoxin, but are usually recovered in the greatest concentration on the water-soluble globulin.

A STUDY OF THE HEMOLYTIC STREPTOCOCCI IN THE THROAT AND IN EMPYEMA

BALDWIN LUCKE
AND
MARION HAGUE REA

From the Cantonment Laboratory, Base Hospital, Camp Zachary Taylor, Ky.

In the winter of 1917 and 1918, infections with hemolytic streptococci were one of the most serious medical problems at this camp as well as at other army camps. At first these infections were intercurrent with measles; later they seemed to constitute an independent epidemic. The respiratory and auditory tracts were chiefly involved, the lesions being "sore throat," bronchopneumonia frequently complicated by empyema, pericarditis and peritonitis and otitis and mastoiditis occasionally complicated by meningitis. Of these various affections, bronchopneumonia was the most important, especially since in over 30% of cases it was accompanied by empyema.

Hamburger and Fox¹ have shown that the epidemic was composed of three variables—measles, streptococcus disease, and pneumococcus disease—and indicated their mutual relations. H. L. Alexander² demonstrated that the streptococcus was a highly responsible factor in this epidemic and widely distributed in the camp, following this up by work in association with Levy³ which went to prove that a streptococcus carrier state was an indication of the probability of complications in measles. Lucke⁴ noted that the pathologic anatomy differed from that of the usual pre-war measles and bronchopneumonia, and presented the results of his postmortem work during the fall and winter before the Camp Taylor Medical Society in April, 1918. These findings were coincident with and confirmed by McCallum.⁵ Gay⁶ has recently summarized the studies from various army camps and clarified the atmosphere about the position of the streptococcus.

The occurrence of hemolytic streptococci in the normal throat, its presence in the throat of measles patients and its relation to acute respiratory diseases have been the subject of numerous studies. In this camp, Levy and Alexander² found the incidence of hemolytic streptococcus carriers among 489 new recruits, as they stepped from the train, to be 14.8%; in 388 measles patients, 77.1%.

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¹ Medical Clinics of North America, December, 1918, p. 322; Jour. Am. Med. Assn., 1918, 70, p. 1758.

² Jour. Am. Med. Assn., 1918, 70, p. 775.

³ Jour. Am. Med. Assn., 1918, 70, p. 1827.

⁴ Jour. Am. Med. Assn., 1918, 70, p. 2005.

⁵ Cole, R., and MacCallum, W. G.: Jour. Am. Med. Assn., 1918, 70, p. 1146. MacCallum, W. G.: The Medical Clinics of North America, December, 1918.

⁶ Jour. Lab. and Clin. Med., 1918, 3, p. 721.

and in 95 apparently healthy men from an organization which had furnished a large number of measles patients, 83.2%.

It is the purpose of this study to ascertain whether the streptococci from such various sources are biologically similar or dissimilar, and whether they are identical with strains isolated from empyema fluids. Kinsella⁷ has shown all hemolytic streptococci to be immunologically identical by the complement fixation test. To further study their relationship, the following criteria have been employed in this work: growth in serum peptone broth, morphology, hemolysin production on blood agar, quantitative hemolysin estimation, carbohydrate reaction, and virulence for rats.

Our present knowledge of the streptococcus group has been so thoroughly recently reviewed by Holman,⁸ Blake,⁹ Gay,⁶ and others, that citations and discussions are here omitted as unnecessary.

MATERIAL AND METHOD OF STUDY

Organisms were isolated from the throats of patients belonging to five disease groups: (1) acute infections of the upper respiratory tract; (2) noninfectious diseases (apparently normal throats); (3) uncomplicated measles; (4) from the pus of patients suffering with empyema preceded by measles, and (5) empyema not preceded by measles.

The throat swab or the pleural pus was streaked on blood-agar plates, colonies picked therefrom, and transferred to blood-agar slants. All studies were made from these as stock cultures. All mediums were placed in the thermostat at 37 C. for 1 hour before using. As far as possible the various tests were made on the same date so as to permit checking up. The general routine was to inoculate horse serum peptone broth and blood plates; record the appearance of growth in both after 18 hours; use the supernatant fluid from the horse serum peptone broth for hemolysin titration and the sediment for morphologic studies. Then carbohydrate mediums were inoculated and rats injected. Practically all tests were repeated to determine any variation in results.

ISOLATION OF STREPTOCOCCI

We had no difficulty in isolating streptococci in pure cultures because of their general occurrence. Throat swabbings on blood-agar plates often gave a pure culture and the pleural fluids contained in every instance a pure growth. After isolation from the original

⁷ Kinsella, R. A., and Swift, H. F.: *Jour. Exp. Med.*, 1918, 28, p. 169.

⁸ *Jour. Med. Research*, 1916, 34, p. 377; *Jour. Infec. Dis.*, 1914, 15, p. 209, and 1914, 15, p. 227.

⁹ *Jour. Med. Research*, 1917, 36, p. 99.

material, cultures were repeatedly plated to insure their purity. It is noteworthy here that smears from the pleural fluids showed such a dense growth of short and long chain streptococci as to resemble the growth in an artificial culture medium. In several instances the empyema patient had received treatment with gentian violet irrigation. The work done in association with the surgical department would seem to indicate that no profit was experienced by the patient from the use of gentian violet, and it was perfectly clear that this dye exerted no inhibitive action on the organisms in the pus, for smears and cultures from such cases presented no variation, and we have kept streptococci for several months in fluids heavily tinged with gentian violet. The pleural fluids were always retained, since we found they sustained the life of the organisms admirably in the icebox. If contamination of any of the stock cultures occurred, we have been able to recover the pure strain by growing it according to Holman's excellent method in horse serum broth, in which the streptococci would outgrow many of the contaminating organisms; the sediment was then streaked on blood-agar plates and typical colonies picked.

Blood Agar.—Heat was applied to 1.5% plain agar (0.4 +) made from beef infusion and sterilized for 3 days by steam, and to each 90 c.c. 10 c.c. of defibrinated human blood was added after cooling the agar to 50 C. After mixture by rotation, plates and tubes were poured therefrom. (The plates were inoculated by streaking a loopful of material along 3-5 parallel lines. After 24 hours' incubation the plates are examined by holding against the light. Typically the line of incubation is marked as a ribbon, consisting of discrete, minute colonies, 3-6 deep, bounded on either side by a rim of hemolysis. This rim may be described as narrow, medium, or wide. Blood-agar slants were inoculated by dipping the loop into the water of condensation and streaking it in a zigzag manner over the slant. On blood agar, pure cultures, after 24 hours' incubation, present typically minute, barely visible, evenly distributed, colorless, discrete colonies. When held against the light hemolysis in the thinner parts of the slant may be seen. Large, easily visible colonies usually indicate contamination. The water of condensation is usually slightly turbid; dense turbidity indicates contamination).

Horse Serum Peptone Broth.—Peptone meat infusion, 2%, was boiled down to 80% its original volume, adjusted to 0.4 +, tubed and sterilized for 3 days in steam. Horse serum was obtained aseptically and heated to 60 C. for 1 hour in the water-bath to destroy antihemolysins. Two c.c. of this was added to each tube containing 8 c.c. of medium, and incubated for 48 hours to insure sterility. We have found this an admirable medium, easily made, of uniform composition and very rarely contaminated. Typically, streptococci produce in this medium a heavy, white sediment consisting of albumin with entangled chains of organisms; older cultures are apt to present a larger amount of sediment. Yellowish, orange or smoky color of supernatant fluid, or viscid stringy consistency of sediment denote contamination.

TABLE 1
SOURCE: THROAT. DISEASE GROUP: ACUTE INFECTIONS UPPER RESPIRATORY TRACT

No.	Disease and Final Outcome*	Date of Cultures	Date of Tests	Bouillon	Morphology	Blood Plate	Erythrocyte Suspension	Lactose	Mannite	Salicin	Virulence for Rats				
											Date	Age	Weight in Gm.	Amount Injected, C C	Results
1	7/1—Tonsillitis, acute. D 7/18	7/1 7/4	7/12	S-D	L-M-R	F-W	0.20 (1 hr.) 0.075 (2 hr.)	+	—	+					
2	7/15—Pharyngitis, acute. D 7/17	7/15	8/15 8/16 9/1	S S-D S-D	L-L-R L-L-R L-L-R	F-M F-M	0.10 0.012 0.014	++ + +	— — —	++ ++ ++	9/1 9/6	young young	35 31	1.0 0.5P	Died 9½ hours Died 21½ hours
3	7/21—Bronchitis, acute. D 7/25	7/21	8/16 8/26	S	L-L-R M-M-R	F-M	0.15	++ +	— —	++ +					
4	7/11—Pharyngitis, acute. D 7/15	7/15	8/16 8/25	S S	M-M-R L-M-R	F-N F-M	0.05 N.H.	— +	— —	++ ++	8/25	young	36	1.0	Survived
5	5/25—Pharyngitis, acute. fracture of tibia. S.C.D. 7/25	5/30	7/12 8/20 9/4	D-S D-S	B-M-R L-M-R	F-W F-N	N.H. 0.012 0.02	++	—	++	9/4 9/6	young young	30 45	1.0 0.5P	Died 9 hours Died 10 hours
6	6/28—Streptococcus carrier. D 6/28	6/28	8/16 8/26	S	M-L-R B-L-R	C-N	0.4	++ +	— —	++ ++					
7	7/17—Tonsillitis, acute. D 7/22	7/17	8/16 8/26	S S-D	L-L-R B-N-R	F-W	0.05 0.014	++ +	— —	— +					
8	7/15—Tonsillitis, acute. D 7/25	7/15	8/15 8/26	S	L-L-R L-L-R	F-M	0.075	++ +	++ —	— +					
9	6/29—Tonsillitis, acute. 7/3—Bronchitis, acute. D 7/7	6/29	7/12 8/26	S	L-L-R B-M-R	F-W	N.H.	++ ++	— —	++ ++					
10	6/4—Tonsillitis, acute. D 6/19	6/19	7/23	D-S	L-M-R	0.0125	+	+	+					

11	6/29—Tonsillitis, acute. D 7/8	6/29 7/4	7/12 8/6	D-S S-D	L-N-E L-M-R	F-W	0.5 0.3 0.3	++	— — ..	++	9/4 9/6 9/6 9/6	young young young adult	62 .. 42 185	1.0 1.0 0.5P 2.0P	Died 6 hours Died 5 hours Died 6½ hours Survived
12	6/28—Bronchitis	6/28 7/3	7/15 7/16	S-D	M-M-R	F-W	0.2 (1 m.) 0.1 (2 m.) 0.05 (1 m.) 0.01 (2 m.)	± — —	— — —	+					
13	7/9—Tonsillitis, acute. D 7/13	7/10	8/15 8/16 9/14	S S	M-M-R M-M-R	F-M F-M	N.H. 0.3	± — +	— — —	+					
14	6/28—Tonsillitis, acute. D 7/3	6/30	7/15 8/29 9/14	S-D D-S	M-L-E	M-M	0.075 (1 m.) 0.05 (2 m.)	+++ +++ +++	+++ +++ +++	+++ +++ +++					
15	7/29—Streptococcus car- rier. D 7/29	7/29	8/15 8/25	S S-D	L-M-R L-L-R	M-M O-M	0.025 0.025	±± +	— —	— +	8/25	young	40	1.0	Died 12 hours

* Disease and Final Outcome: The disease is preceded by the date of development; the date following is that of final disposition. D = indicated return to duty; S.O.D. = discharged from army on Surgeon's certificate of disability; D.I. = died; T.G.H. = transferred to general hospital.

Bouillon: S = sedimentation; S-D = sedimentation with slight diffuse turbidity; D-S = diffuse turbidity with slight sedimentation.

Morphology: The first letter indicates length of chain; L = longus; M = medius; B = brevis. The second letter indicates size of individual cocci: L = large; M = medium; S = small. The third letter indicates shape of individual cocci: R = round; F = elongated.

Blood Plate: The first letter indicates size of colony: F = fine (minute); O = coarse. The second letter indicates width of hemolytic rim: N = narrow; M = medium; W = wide.

Carbohydrate Fermentations: + indicates fermentation; —, no fermentation; ±, doubtful fermentation.

Morphology.—Smears were made from 18-hour horse serum peptone broth culture, stained by Gram's method, and the length of chain and size and shape of the individual cocci recorded.

Carbohydrate Broth.—Peptone meat infusion (2%) 0.4 + containing 1% of mannite, lactose, and salicin, respectively, with Andrade's indicator were used. To each tube 0.2 cc of an 18-hour horse serum broth culture was added and the tubes incubated for 5 days. In many instances smears were made from the sediment to detect contamination. Doubtful reactions were repeated.

Streptolysin.—Hemolytic streptococci produce certain soluble toxins of which a hemolysin (streptolysin) is the most important. Its properties may be summarized as follows: Streptolysin production takes place best in serum containing liquid mediums, beginning a few hours after incubation, reaching its maximum in from 16-18 hours, gradually decreasing, and disappearing after 48 hours. It is thermolabile, heating for 30 minutes at 55 C. destroying it. After centrifugating a streptococcus culture, the supernatant clear fluid is as potent as the bacterial suspension. Filtration through a Berkefeld, or similar filter, slightly decreases its potency. McLeod¹⁰ attributes much importance to streptolysin and believes that the amount produced by a given strain stands in direct relation to its virulence. Quantitative estimation of hemolysin has been used by McLeod,¹⁰ Lyall,¹¹ and others, adding to a standard erythrocyte suspension increasing quantities of streptolysin and incubating the mixture for a certain time. The smallest amount of streptolysin which produces complete solution of the red corpuscles is taken as the unit and called by McLeod the "minimal hemolytic dose." Since practically all the components of this test are of variable nature, we have aimed to standardize each substance so as to permit comparison of the results.

The supernatant fluids from 18-hour cultures in horse serum peptone broth were used ("streptolysin solution"). We found that the amount of streptolysin produced does not depend on the number of bacteria used for seeding the serum broth, provided that a sufficient number be inoculated to cause growth. Thus, we introduced from 1-10 drops, respectively, of a suspension of streptococci into a series of 10 serum broth tubes. After 18 hours' incubation, practically an identical hemolytic titer was found in each tube. One loopful of streptococci from a blood-agar slant is sufficient to produce maximum growth in horse serum broth. Maximum hemolysin production occurred invariably in mediums affording suitable conditions for luxuriant growth provided carbohydrates were not present. In ordinary peptone broth, hydrocele fluid, or broth of low serum content, streptococci grow poorly and a corresponding low feeble hemolysin production obtained.

The sheep corpuscle suspension was made so that each 1 cc would contain 300,000 erythrocytes (this is approximately a 2.5% suspension). A measured quantity of sheep blood was collected with a syringe previously rinsed with sodium citrate solution, and washed 3 times with normal salt solution. A suspension in salt solution approximating 3% was then made, and the cells counted with a white blood pipet. By a simple calculation and the addition of salt solution the standard suspension was obtained and checked up by another cell count. This suspension was always used on the same date.

The "streptolysin solution" in amounts ranging from 0.01-1 cc was introduced into a series of test tubes, 1 cc of the erythrocyte suspension added to

¹⁰ Jour. Path. and Bacteriol., 1915, 19, p. 392.

¹¹ Jour. Med. Research, 1914, 30, pp. 487 and 515.

TABLE 2

SOURCE: THROAT. DISEASE GROUP: NONINFECTIOUS DISEASES

No.	Disease and Final Outcome	Date of Cultures	Date of Tests	Bouillon	Morphology	Blood Plate	Erythrocyte Suspension	Lactose	Mannite	Salicin	Virulence for Rats				
											Date	Age	Weight in Gm.	Amount Injected, C C	Results
1	7/8—Myalgia. D 7/14	7/ 8	7/23	D-S	L-M-R	F-N	0.2	+	—	+					
2	7/2—Ulcer of nasal septum. D 7/10	7/ 2	7/23 9/23	D-S	M-S-E	F-W	N.H.	+	—	+					
3	6/13—Pulm. tuberculosis, chronic. T.G.H.	7/ 3	7/ 9	S-D	M-M-R	F-N	0.012	+	—	+					
4	7/13—Reaction to typhoid vaccine. D 7/17	7/14	8/13 8/26 9/24	D-S	L-L-R M-L-R	F-N	0.0125	± + +	+	+					
5	7/16—Neurocirculatory asthenia. D 7/20	7/16	8/13 9/ 1	S-D S-D	L-M-E L-L-R	F-M	0.0125 0.01	+	—	+	9/1 9/2	young young	30 45	1.0 1.0	Died 35½ hours Died 20 hours
6	7/8—Reaction typhoid vaccine. D 7/10	7/ 8	8/15 8/26	S	L-L-R M-M-R	F-M F-M	0.025	± +	—	+					
7	7/15—Cardiac disorder. D 7/25	7/15	8/15 8/20	S	M-M-R	F-M	0.05	± +	—	+	9/20	mouse	15	0.2	Died 26 hours
8	7/3—Gastric ulcer. S.C.D. 8/8	7/15	7/15 7/16	S-D D-S	M-M-R	M-W	N.H. 0.15 (1 hr.) 0.05 (2 hr.)	+	+	+					
9	7/14—Gastric ulcer. S.C.D. 8/25	7/14	8/15 9/ 4	S	M-L-R	C-M	0.3 0.08	+	—	+	9/4 9/6 9/6 9/6	young young young young	46 .. 37 180	1.0 1.0 0.5P 2.0P	Died 5½ hours Died 5 hours Died 4½ hours Died 6 hours
10	7/12—Synovitis, acute, of ankles. D 8/9	7/13	8/15 9/14	S	L-L-R	F-M F-M	0.012	± +	—	±					
11	7/8—Epilepsy S.C.D. 8/14	7/ 8	8/15 9/17	S	L-L-R	C-M	0.05	—	—	+					
12	6/13—Malaria D 7/12	6/30	8/11 9/ 4	S-D	M-M-R	F-W	0.075 (1 hr.) 0.025 (2 hr.) 0.03	+	—	+	9/4 9/6 9/6	young young young	59 .. 42	1.0 1.0 0.5P	Died 6 hours Died 5½ hours Died 6½ hours
13	5/9—Injury to cervical vertebral. S.C.D. 7/22	7/ 3	7/11 7/13	D-S	M-M-R	F-W	0.2 (1 hr.) 0.025 (2 hr.) 0.3	+	—	+					

each tube, gently shaken, and the result read after 1 hour's incubation in the water-bath at 37 C. In several instances second readings were taken after 2 hours; as a general rule the hemolysis was doubled in this time. Usually the dilution was not carried below 0.01 c.c. Streptolysin solutions which were not hemolytic in quantities of 1 c.c. were designated N H.

Virulence for Rats.—Virulence observations were made on cultures younger than the 4th generation. Small white rats, 6 weeks old, approximately of the same weight, and large rats 3 months old, were used. All injections were made intraperitoneally with a well shaken 18-hour horse serum broth culture. A necropsy was made usually within an hour after death, smears and cultures taken from the peritoneal exudate and heart blood; streptococci in pure culture were recovered in every instance. Hemolytic titrations were conducted with these "passage" cultures in several instances.

RESULTS

The results are summarized in tables 1-5 and are here discussed collectively.

Growth in Horse Serum Peptone Broth.—As a general rule, the growth was a heavy, white, flocculent sediment with faintly opalescent supernatant fluid. Less often the supernatant fluid was cloudy; diffuse clouding with slight sediment occurred least frequently. No differences were noted in the strains from the various sources or disease groups. The appearance of the culture often varied at different tests; thus, a strain which usually presented a distinct sediment without any clouding, would occasionally exhibit distinct clouding and only slight sedimentation.

Morphology.—The length of the chains and the size of the individual cocci did not present many characteristic features and often varied from week to week. This variation usually accompanied changes in the appearances of the broth culture. The shape of the individual cocci remained constantly as a slightly laterally compressed oval. Elongated forms were only rarely met with.

The tables illustrate well that no reliance can be placed on morphologic features. No essential difference was noted in the various strains.

Growth on Blood Plates.—The width of the hemolytic rim appeared to depend on the consistency and amount of the culture used for inoculating, on size and shape of loop, on consistency of the culture medium, the pressure of the loop in inoculating the medium and the luxuriance of the growth. Contaminating organisms, such as staphylococci, are usually much larger than the streptococcic colonies, and

TABLE 3
SOURCE: THROAT. DISEASE GROUP: MEASLES UNCOMPLICATED

No.	Disease and Final Outcome	Date of Cul- tures	Date of Tests	Bouil- lon	Mor- phology	Blood Plate	Erythro- cyte Suspend- sion	Lac- tose	Man- nite	Sali- cin	Virulence for Rats				
											Date	Age	Weight in Gm.	Amount Injected, C C	Results
1	5/25—Measles, D 7/16	7/ 2 7/ 9	8/13 8/26 9/14	D-S	L-M-R L-M-R	M-M F-M	0.0125	± + +	+ + +	+					
2	6/4—Epilepsy; 7/8—Measles, S.C.D. 7/28	7/16	8/30	S	L-L-R	F-M	0.4	+	—	+					
3	6/25—Measles, D 7/10	7/10	8/15 8/26	S	L-L-R M-L-R	F-W	0.012	± +	— —	+					
4	7/10—Measles, D 8/7	7/10 7/16	8/14 9/12 9/17	S	L-L-R	F-M	0.4	— — —	— — —	± + +					
5	7/13—Measles, D 8/13	7/13	8/15 8/27	S	L-L-R L-L-R	F-M F-W	0.4	— +	— —	— +					

TABLE 4
SOURCE: PLEURAL FLUID. DISEASE GROUP: EMPYEMA, PRECEDED BY MEASLES

No.	Disease and Final Outcome	Date of Cultures	Date of Tests	Bouillon	Morphology	Blood Plate	Erythrocyte Suspension	Lactose	Mannite	Salicin	Virulence for Rats				
											Date	Age	Weight in Gm.	Amount Injected, C C	Results
1	4/20—Measles; 5/18—Bronchopneum.; 5/22—Empyema; 5/27—Thoracotomy. H 9/1	5/13 5/25	8/19 9/14 9/20	S	L-S-R	F-M	0.025	± — +	— + +	++ ++ +	9/4 9/6	young young	54 ..	1.0 1.0	Died 6 hours Died 5½ hours
2	4/10—German measles; 5/1—Bronchopneum.; 5/7—Empyema; 6/3—Thoracotomy. H 9/1 H 9/1	5/5 5/9	7/16 8/19 9/1	D-S S-D S-D	M-M-R L-M-R B-L-R	F-M	N.H. 0.025 0.028	++	—	++	9/1 9/6 9/6	young young adult	42 40 187	1.0 0.5P 2.0P	Died 9 hours Died 11 hours Died 10½ hours
3	3/29—Measles; 4/18—Tonsillitis; 4/19—Bronchopneum.; 4 21—Empyema; 4 24—Thoracotomy. Dd. 5/4	4/24 4 25	7/23 8 19 9 1 9 4	D-S S	L-M-R B-M-R	F-M C-M	0.025 0.007 0.01 (P)	++ + ..	— — ..	++ ++ ..	9/1	young	53	1.0	Died 9½ hours
4	3/29—Measles; 4/12—Bronchopneum.; 4/24—Empyema; 5/2—Thoracotomy. S.C.D. 8/11	4/24	8/6 9 3 9/17	S S-D	L-L-R L-M-R	F-W	N.H. 0.02	— ± ±	— — —	++ ++ +	9/1 9/6	young young	40 32	1.0 0.5P	Died 10 hours Died 30 hours

of an opaque, white color. Sometimes, however, individual streptococcic colonies are larger than usual, broad and flat. This is commonly met with in cultures which have stood for a long period of time in the icebox, or which have been overincubated. Hazy, ground glass hemolysis is produced when incubation is carried on for less than 24 hours, at a lower temperature than 37 C. on unsuitable mediums or in old cultures. The size of the colonies was usually small, the rim of hemolysis generally medium in size. No constant uniform variation in hemolysis occurred on blood-agar plates such as could not be explained on technical grounds. There was also no constant variation in the size or type or quality of colony in the five groups discussed.

Quantitative Hemolysin Determination.—All strains exhibited a high hemolytic power. This, while slightly varying at times, was as a rule fairly constant for the individual strains. Variations of hemolytic potency were usually associated with variations in bouillon growth and morphology, although no definite law could be formulated. Cultures, after passage through one animal, exhibited practically no change in titers from those of the original cultures; however, further passage was not performed. In a general way it would seem to us that hemolysin production depended on favorable conditions for growth and that any given strain, having a low hemolytic degree, exhibited decided increase in hemolysin production on reculture and repetition of the test. This was equally true of throat and pleural strains. Thus, for instance, there are several strains that proved nonhemolytic in quantities of 1 c.c. which later on showed a high degree of hemolysis. No essential changes were noticed among the various strains, so that if the hemolytic property of a strain of streptococcus stands in any relation to virulence, it would seem that the organisms isolated from the throat were as virulent and as capable of cellular destruction as the strains from the pleural exudate.

Carbohydrate Fermentation Reaction.—The tables show, in several instances, decided variation in carbohydrate reaction. This may be due to accidental contamination or to the presence of more than one strain of streptococcus. We urge, therefore, repetition of all carbohydrate tests preferably after an interval. This we have done in many instances; in conflicting readings, the last result obtained being the one recorded. The results are summarized in table 6. It shows that according to Holman's classification *Streptococcus pyogenes* pre-

TABLE 5
SOURCE: PLEURAL FLUID. DISEASE GROUP: EMPYEMA, NOT PRECEDED BY MEASLES

No.	Disease and Final Outcome	Date of Cul- tures	Date of Tests	Bouil- lon	Mor- phology	Blood Plate	Erythro- cyte Suspend- sion	Lac- tose	Man- nite	Sali- cin	Virulence for Rats				
											Date	Age	Weight in Gm.	Amount Injected, C C	Results
1	Pneumonia lobar; empyema. S.C.D. 3/5	12/17	7/25	S-D	L-L-R	F-W	N.H.	+	—	±					
2	4/16—Empyema; thoracotomy. D 8/15	4/ 8 4/11	7/26 8/20 9/ 4	S-D S	B-M-R L-L-R	N-W C-W	0.075 0.012 0.03	+	—	+	9/4 9/6	young young	53 ..	1.0 1.0	Died 7½ hours Died 6½ hours
3	5/ 5—Bronchopneum.; 5/ 7—Empyema; 5/29—Thoracotomy. H 9/1	5/ 9 5/12	8/ 9 8/20	L-M-R L-L-R	F-M	0.05 0.10	+	—	+					
4	4/23—Arthritis, acute; 5/ 3—Pleurisy; 5/ 5—Empyema. H 9/1	5/ 5 5/20 5/26	7/16 7/22 8/18 8/20	S S-D S-D S-D	L-M-R L-M-R L-L-R L-M-R	F-M F-M F-M F-M	0.05 0.025 0.025	— — —	++ ++ +	++ ++ +					
5	4/ 8—Bronchitis; 4/17—Empyema; 4/25—Thoracotomy. S.C.D. 8/25	5/15 5/18	8/ 6 9/ 1	D-S S-D	L-M-R M-L-R	F-M F-M	0.01 0.05	++	—	++	9/1 9/4	young young	45 40	1.0 0.1	Died 12 hours Survived
6	4/ 8—Bronchitis; 4/17—Empyema; 4/25—Thoracotomy. S.C.D. 8/15	4/17 5/18 5/21	8/19	S	L-S-R	F-M	N.H.	+	—	+	9/20	mouse	17	0.2	Died 7½ hours

7	4/13—Tonsillitis; 4/17 Empyema. Dd. 4/21	4/17	8/16 9/ 4	D-S S-D	B-M-R B-L-R	0.012 0.01	+	—	+					
8	4/ 8—Pleurisy; 4/15—Empyema. Dd. 4/16	4/15 4/16	7/11 8/19 8/25 9/ 1	D-S S S-D D-S	B-M-R L-S-R M-M-R L-L-R	F-W C-M	N.H. 0.075 0.01 0.014	+	—	—	+	+	+	+	Living
9	4/ 7—Empyema; 4/17—Thoracotomy.	4/ 8	7/11 7/12 8/20	D-S D-S	L-L-R B-M-R L-M-R	S-W F-W	0.05 (1 m.) 0.03 (2 m.) N.H.	+	—	+					
10	5/ 1—Bronchitis, acute; 5/ 1—Empyema; 5/28—Thoracotomy. S.C.D. 9/11 S.C.D. 9/11 S.C.D. 9/11 S.C.D. 9/11	5/ 8 5/ 9	7/16 8/19 8/21 8/16 8/19 7/16 8/19 9/ 1 9/ 2	S-D S S S S S S-D S-D	B-M-R B-S-R L-S-R M-M-R L-M-R B-M-R B-L-R B-L-R	F-M F-M F-M F-W F-N F-M F-M 0.005 (P)	0.3 0.05 0.05 0.05 0.25 0.012 0.014 0.005 (P)	+	—	+	+	+	+	+	Died 8½ hours Survived
11	5/ 1—Bronchitis, acute; 5/ 1—Empyema; 5/28—Thoracotomy. S.C.D. 9/11	5/26	7/22 8/19 9/ 1	S-D S S-D	B-M-R M-M-R B-L-R	F-N	0.25 0.007	+	—	+	+	+	+	+	Died 8 hours Died 7 hours Died 6½ hours Died 10 hours Died 21 hours Survived

dominated in all disease groups; *Streptococcus infrequens* occurring next; *Streptococcus equi* occurred only twice and that in the throat; *Streptococcus hemolyticus* II was found once in a pleural exudate. The other four types of Holman—*Streptococcus hemolyticus* I and III, *subacidus*, and *anginosus*—did not occur in our series. There was no essential difference in hemolysin production by the strains of the four types found, nor did they exhibit any cultural or morphologic distinctions.

Virulence for Rats.—No difference in the virulence for rats of the various disease groups was apparent. In practically all instances the animals died within 24 hours, the majority within 6-12 hours, thus

TABLE 6
SUMMARY OF CARBOHYDRATE FERMENTATION TESTS

Source and Disease Group	<i>Streptococcus</i> <i>Infrequens</i>	<i>Streptococcus</i> <i>Pyogenes</i>	<i>Streptococcus</i> <i>Hemolyti-</i> <i>eus</i> II	<i>Streptococcus</i> <i>Equi</i>
Throat:				
Acute infectious diseases.....	2	13	—	—
Noninfectious diseases.....	1	11	—	1
Measles.....	1	3	—	1
Empyema:				
Preceded by measles.....	1	4	—	—
Not preceded by measles.....	—	12	1	—
Total.....	5	43	1	2

showing our strains to be highly virulent. If hemolysin production stands in intimate relation to virulence, this high virulence to rats would confirm our findings of a uniform high hemolytic titer of the various strains.

CONCLUSIONS

Streptococci isolated from the throat and from empyema exudate during the epidemic of the winter of 1917 and 1918, appeared biologically identical and highly virulent as based on the criteria mentioned in the early part of the paper. Our study confirms the observations of others, using immunological procedures, and gives additional support to the belief that the *streptococcus* carrier state is an indication of the possibility of complications in respiratory tract diseases.

SODIUM OXY-MERCURY-ORTHO-NITRO PHENOLATE (MERCUROPHEN)

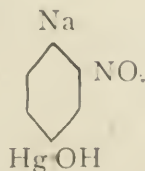
WITH SPECIAL REFERENCE TO ITS PRACTICAL VALUE
AS A DISINFECTANT

JAY F. SCHAMBERG, JOHN A. KOLMER, GEORGE W. RAIZISS
WITH THE ASSISTANCE OF
MARY E. TRIST

*From the Dermatological Research Laboratories, Philadelphia **

In a preliminary report¹ we briefly described the properties of a mercurial compound, elaborated during a systematic investigation in the chemotherapy of mercurial compounds, which was found to possess certain superior antiseptic and germicidal properties and to which the name of "mercurophen" was given. Since then additional laboratory and clinical studies have been conducted on a large scale and the object of this article is to give in a brief manner a further report on the properties of this compound and its value as a disinfectant. The additional work has confirmed our earlier results and the superiority of mercurophen over other mercurials, notably mercuric chlorid, which was taken as a standard for comparison, and in addition it has been shown that the new compound possesses a marked affinity and destructive influence for staphylococci and spore-forming organisms and also possesses other distinctive features of much interest and importance from the viewpoint of chemotherapy.

Mercurophen has been designated chemically as sodium oxymercuryorthonitrophenolate, containing 53% mercury and having the following structural formula:



The exact position of mercury in the molecule is not definitely demonstrated.

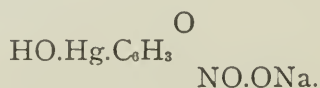
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¹ Schamberg, J. F.; Kolmer, J. A., and Raiziss, G. W.: Jour. Am. Med. Assn., 1917, 68, p. 1458.

It is a brick red powder, freely soluble in hot water, and capable of being molded into tablets, likewise freely soluble; solutions may be prepared as concentrated as 1:100 but not usually beyond this degree. The solutions are deep amber in color, which is distinctive in dilutions as high as 1:10,000 or more. Concentrated solutions, as 1:500 and 1:1,000, after standing several days may show a slight amount of reddish sediment but solutions maintain an even germicidal potency over long periods of time providing evaporation does not occur. The powder and its solutions are odorless; solutions of 1:1,000 have a slightly bitter metallic taste while higher dilutions, as 1:5,000 and 1:10,000, are practically tasteless.

As previously stated mercurphen was prepared during a study and elaboration of compounds of mercury and phenol; when our preliminary report was made we were not aware that this compound had been briefly described by Häntzsch and Auld² under the name of "merkurinitrophenole," the sodium salt of mercury hydrate aci-ortho-nitrophenol with a theoretical sodium content of 6.1 and the following structural formula:



The authors referred to made no biological studies of this compound, and further references to it have not been found.

THE ANTISEPTIC OR BACTERIOSTATIC PROPERTIES OF MERCUROPHEN

As is well known very slight modifications in technic may alter the results of germicidal tests; for this reason we have sought to employ simple methods along well known lines and to present in this paper the essentials of each method employed.

The antiseptic or bacteriostatic (a term proposed by Basil Gildersleeve) test yields very sharp, decisive and easily read results concerning the power of a substance to inhibit multiplication of a micro-organism, although actual killing or permanent destruction and crippling are not determined unless subcultures are made for viable organisms; as a general rule, we have found with *B. typhosus* and staphylococci that there is a distinct relationship between the bacteriostatic and germicidal values of a substance under study although with spore bearing bacteria as *B. anthracis* and *B. subtilis*, a compound may possess marked bacteriostatic and feeble germicidal activity.

² Bericht. Deutsch. Chem. Gesellsch., 1906, 39, p. 1105.

In making these tests dilutions of mercuriofen and other germicidal substances were made from stock 1:1,000 solutions with sterile distilled water and 1 cc of each dilution placed in a series of sterile test tubes; dilutions of phenol were prepared in a similar manner from a standardized 5% solution. The test organism was grown in broth for 48 hours, shaken with beads to break up clumps and 1 cc added to 100 cc of plain neutral beef extract broth of which 5 cc was added to each test tube, making the total volume 6 cc. In this manner we were sure that each tube was seeded with the same number of bacteria, and owing to the simplicity of the technic errors were reduced to a minimum. In each experiment controls of the cultures were included; the results were recorded after 5 days' incubation in a thermostat. In numerous experiments subcultures were made of the contents of the tubes showing no evidence of growth, by transferring a 4 mm. loopful to a slant of plain neutral agar.

TABLE 1
RESULTS OF ANTISEPTIC TESTS WITH *B. TYPHOSUS*

Substance	Per-centage Pure Hg	Final Dilutions and Results									
		1:300,000	1:360,000	1:420,000	1:480,000	1:540,000	1:600,000	1:1,200,000	1:1,800,000	1:2,400,000	1:3,000,000
Mercuriofen No. 15.....	53	—	—	—	+	+	+	+	+	+	+
Mercuriofen No. 16.....	47	—	—	+	+	+	+	+	+	+	+
Mercuric chlorid.	74	—	—	—	—	—	+	+	+	+	+
Hg Oxycyanid...	83	—	—	—	—	—	—	+	+	+	+
Hg Cacodylate..	20	+	+	+	+	+	+	+	+	+	+
Hg Succinimid...	50	—	—	—	—	—	+	+	+	+	+

— = no growth; + = growth.

TABLE 2
RESULTS OF ANTISEPTIC TESTS WITH *STAPHYLOCOCCUS AUREUS*

Substance	Per-centage Pure Hg	Final Dilutions and Results									
		1:300,000	1:360,000	1:420,000	1:480,000	1:540,000	1:600,000	1:1,200,000	1:1,800,000	1:2,400,000	1:3,000,000
Mercuriofen No. 15.....	53	—	—	—	—	—	—	—	—	—	+
Mercuriofen No. 16.....	47	—	—	—	—	—	—	—	—	+	+
Mercuric chlorid.	74	—	—	—	—	—	+	+	+	+	+
Hg Oxycyanid...	83	—	—	—	—	—	—	+	+	+	+
Hg Cacodylate..	20	+	+	+	+	+	+	+	+	+	+
Hg Succinimid...	50	—	—	+	+	+	+	+	+	+	+

— = no growth; + = growth.

The Rawling strain of *B. typhosus* was employed; the strain of *staphylococcus aureus* was obtained from the pus of a furuncle and the strain of *B. anthracis* from a malignant pustule, about 2 years ago.

The results of several experiments with *B. typhosus*, *staphylococcus aureus* and *B. anthracis* and different lots of mercuriofen and a number of well known mercurials are shown in tables 1, 2 and 3;

a summary of the general results are shown in table 4 in which the highest antiseptic or bacteriostatic dilutions are given with a comparison of the mercurial compounds with phenol as expressed in the "phenol coefficients," and a comparison of the mercurorphen and other mercurial compounds with mercuric chlorid, as expressed in the column under "mercuric chlorid coefficients."

TABLE 3
RESULTS OF ANTISEPTIC TESTS WITH B. ANTHRACIS

Substance	Per- cent- age Pure Hg	Final Dilutions and Results									
		1: 300,000	1: 360,000	1: 420,000	1: 480,000	1: 540,000	1: 600,000	1: 1,200,000	1: 1,800,000	1: 2,400,000	1: 3,000,000
Mercurorphen No. 15.....	53	—	—	—	—	—	—	—	—	+	+
Mercurorphen No. 16.....	47	—	—	—	—	—	—	—	+	+	+
Mercuric chlorid.	74	+	+	+	+	+	+	+	+	+	+
Hg Oxyeyanid...	83	+	+	+	+	+	+	+	+	+	+
Hg Cacodylate...	20	+	+	+	+	+	+	+	+	+	+
Hg Succinimid...	50	+	+	+	+	+	+	+	+	+	+

— = no growth; + = growth.

TABLE 4
SUMMARY OF RESULTS OF ANTISEPTIC TESTS; PHENOL AND MERCURIC CHLORID COEFFICIENTS

Substance	Per- cent- age Pure Hg	Highest Antiseptic Dilution after Incubation for 5 Days at 37 C.			Phenol Coefficients			Mercuric Chlorid Coefficients		
		B. ty- phosus	Staph. aureus	B. an- thraxis	B. ty- phosus	Staph. aureus	B. an- thraxis	B. ty- phosus	Staph. aureus	B. an- thraxis
Mercurorphen No. 4.....	53	1: 500,000	1:1,200,000	1:1,200,000	714	1714	2500	-0.92	+2.2	+4.3
Mercurorphen No. 6.....	53	1: 480,000	1:1,200,000	1:1,000,000	685	1714	2083	-0.9	+2.2	+3.5
Mercurorphen No. 11.....	53	1: 480,000	1:1,800,000	1:1,400,000	685	2572	2916	-0.9	+3.3	+5.0
Mercurorphen No. 12.....	53	1: 420,000	1:1,800,000	1:1,200,000	600	2572	2500	-0.8	+3.3	+4.3
Mercurorphen No. 15.....	53	1: 420,000	1:2,400,000	1:1,800,000	600	3428	3666	-0.8	+4.4	+6.4
Mercurorphen No. 16.....	47	1: 360,000	1:1,800,000	1:1,200,000	514	2572	2500	-0.66	+3.3	+4.3
Hg Oxyeyanid...	83	1:1,200,000	1: 600,000	1: 280,000	1714	857	583	+2.2	+1.1	1.0
Hg Succinimid...	50	1: 540,000	1: 360,000	1: 250,000	771	514	520	1.0	-0.66	-0.9
Hg Cacodylate...	20	1: 180,000	1: 120,000	1: 100,000	257	171	208	-0.33	-0.2	-0.4
Phenol.....	0	1: 700	1: 700	1: 480	0	0	0	0	0	0
Mercuric chlorid.	74	1: 540,000	1: 540,000	1: 280,000	771	771	583	0	0	0

These results may be summarized as follows:

1. The bacteriostatic value of mercuric chlorid and other mercurial compounds for all three of the test organisms bears a direct relationship to their content in mercury and probably to the degree of

electrolytic dissociation. For example, a mercuric chlorid containing 74% mercury proved bacteriostatic for *B. typhosus* in dilution of 1:540,000, while the oxycyanid of mercury containing 83% mercury proved bacteriostatic in dilutions as high as 1:1,200,000, the caco-dylate—which showed in our analysis but 20% mercury—was bacteriostatic in only 1:180,000. The same relationship between content of mercury in mercuric chlorid and other mercurials and bacteriostatic activity was shown in experiments with *staphylococcus aureus* and *B. anthracis*.

2. Phenol showed the same bacteriostatic activity for both *B. typhosus* and *staphylococcus aureus* being about 1:600 and 1:480 for anthrax bacilli and *B. subtilis*.

3. Mercurophen is more bacteriostatic for staphylococci and anthrax bacilli than mercuric chlorid and other of the ordinary mercury compounds and this constitutes an important observation from the chemotherapeutic and practical standpoint. For example, mercurophen containing 53% mercury was bacteriostatic for staphylococci in dilutions varying from 1:1,200,000 to 1:2,400,000, while mercuric chlorid containing 74% mercury was bacteriostatic in 1:540,000, and the oxycyanid containing as much as 83% mercury in but 1:600,000. With the anthrax bacillus a similar superiority of mercurophen was noted inasmuch as this compound was bacteriostatic in about 1:1,400,000 whereas mercuric chlorid was bacteriostatic in but 1:280,000.

As shown in table 4 under mercuric chlorid coefficients, mercurophen was somewhat less bacteriostatic for *B. typhosus* than mercuric chlorid being from -0.8 to -0.92 calculated on the basis of mercuric chlorid as 1.0, while with the *Staph. aureus* and *B. anthracis*, mercurophen gave indices varying from $+2.2$ to $+6.4$ as calculated on the same basis.

4. As compared with phenol, mercurophen was generally from 514 to 714 times more bacteriostatic for *B. typhosus* while mercuric chlorid was 771 times more bacteriostatic; with *Staph. aureus* the mercurophens gave coefficients varying from 3,428 to 1,714 as compared with 771 shown by mercuric chlorid. With *B. anthracis*, mercurophen showed a bacteriostatic phenol coefficient of 2,000 to 3,600 as compared to a coefficient of 600 by mercuric chlorid.

RELATION OF ANTISEPTIC TO GERMICIDAL ACTIVITY

As previously stated, the antiseptic values of the substances studied were recorded in this method after a period of incubation for 5 days; the highest antiseptic values were observed at the end of the first 24 hours' incubation but from then to the end of 5 days, growths usually appeared in the next (one or more) of the lower dilutions. With the completion of the 5 day period subcultures of the clear or negative tubes showing antiseptic, restraining or bacteriostatic activity made by transferring two 4 mm. loopfuls of clear broth to slants of agar, usually showed the presence of viable staphylococci and typhoid bacilli in at least several dilutions lower than the bacteriostatic dilution; for example, mercurophen proved antiseptic for *B. typhosus* in dilution of 1:600,000 and germicidal in 1:360,000; mercuric chlorid was antiseptic in dilution of 1:540,000 and germicidal in 1:420,000; phenol was antiseptic in dilution of 1:660 and germicidal in 1:540. With *B. anthracis* and *B. subtilis* the differences were more striking; for example, phenol proved antiseptic for *B. subtilis* in dilution of 1:480 but germicidal in less than 1:60. With the exception of the spore-forming bacilli, there was found with this technic a definite relation between the antiseptic and germicidal values of the substances under study, and since the technic of the antiseptic test is simple and the results sharp and easily read, we have adopted this technic for determining comparative antibacterial properties of substances in the course of our chemotherapeutic studies.

TABLE 5
GERMICIDAL ACTIVITY OF PHENOL AND MERCUROPHEN FOR *B. TYPHOSUS* IN THE
HYGIENIC LABORATORY METHOD

Substance	Dilution	Time Culture Was Exposed to Action of Disinfection in Minutes						Phenol Coefficient
		2½	5	7½	10	12½	15	
Phenol	1:70	—	—	—	—	—	—	70 40,000 = 571.4
	1:80	+	+	—	—	—	—	
	1:90	+	+	+	—	—	—	
	1:100	+	+	+	+	+	+	
	1:110	+	+	+	+	+	+	
Mercurophen No. 12 Hg 53%	1:40,000	—	—	—	—	—	—	90 120,000 = 1333.3 2 1904.7 952.3
	1:60,000	+	—	—	—	—	—	
	1:80,000	+	+	—	—	—	—	
	1:100,000	+	+	+	+	—	—	
	1:120,000	+	+	+	+	+	—	
	1:160,000	+	+	+	+	+	+	

Beef infusion broth +0.5.

TABLE 6

RESULTS OF GERMICIDAL TESTS WITH B. TYPHOSUS BY THE HYGIENIC LABORATORY METHOD

Substance	Dilution	Time Culture Was Exposed to Action of Disinfection in Minutes						Phenol Coefficient
		2½	5	7½	10	12½	15	
Mercuriophen No. 16 Hg 47%	1:30,000	—	—	—	—	—	—	70 30,000
	1:40,000	+	+	—	—	—	—	90 60,000
	1:50,000	+	+	—	+	—	—	
	1:60,000	+	+	+	+	+	—	
	1:80,000	+	+	+	+	+	+	
	1:100,000	+	+	+	+	+	+	
								2 1105.1
								552.5
Mercuriophen No. 6 Hg 53%	1:40,000	—	—	—	—	—	—	70 40,000
	1:60,000	+	—	—	—	—	—	90 160,000
	1:80,000	+	+	—	—	—	—	
	1:100,000	+	+	+	+	—	—	
	1:120,000	+	+	+	+	+	—	
	1:160,000	+	+	+	+	+	—	
								2 2349.1
								1174.5
Mercuric Chlorid Hg 74%	1:40,000	—	—	—	—	—	—	70 40,000
	1:60,000	+	—	—	—	—	—	90 100,000
	1:80,000	+	—	+	—	—	—	
	1:100,000	+	+	+	+	+	—	
	1:120,000	+	+	+	+	+	+	
	1:160,000	+	+	+	+	+	+	
								2 1682.5
								841.2
Mercury Benzoate Hg 45%	1:30,000	—	—	—	—	—	—	70 30,000
	1:40,000	+	—	—	—	—	—	90 80,000
	1:60,000	+	+	+	—	—	—	
	1:80,000	+	+	+	—	+	—	
	1:100,000	+	+	+	+	+	+	
	1:120,000	+	+	+	+	+	+	
								2 1317.3
								658.6
Mercury Cacodylate Hg 20%	1:10,000	—	—	—	—	—	—	70 10,000
	1:20,000	+	+	—	—	—	—	90 30,000
	1:30,000	+	+	—	+	—	—	
	1:40,000	+	+	+	+	+	—	
	1:60,000	+	+	+	+	+	+	
	1:80,000	+	+	+	+	+	+	
								2 476.1
								238.0
Mercury Sozoidolate Hg 32%	1:20,000	—	—	—	—	—	—	70 20,000
	1:30,000	+	+	—	—	—	—	90 60,000
	1:40,000	+	+	—	+	—	—	
	1:60,000	+	+	+	+	+	—	
	1:80,000	+	+	+	+	+	+	
	1:100,000	+	+	+	+	+	+	
								2 952.3
								476.1

Plain neutral beef extract broth.

THE GERMICIDAL PROPERTIES OF MERCUROPHEN AS DETERMINED BY
THE HYGIENIC LABORATORY METHOD

In conducting these tests the Rawling strain of *B. typhosus* was employed and the technic was exactly as described by Anderson and McClintic³ except that the beef infusion broth employed had an end reaction of +0.5 instead of +1.5, inasmuch as broth with an end reaction of +1.5 did not yield good growths of typhoid bacilli. Parallel tests were made in exactly the same manner with beef extract broth neutral to phenolphthalein inasmuch as this medium was previously employed by us in the Rideal-Walker tests. The dilutions of phenol were prepared from a 5% solution obtained from the Hygienic Laboratory and also from our own standardized solution varying not more than 0.03% from the standard 5% solution. Subcultures were made with the regulation 4 mm. loop standardized in the Hygienic Laboratory.

TABLE 7

SUMMARY OF RESULTS OF GERMICIDAL TESTS WITH *B. TYPHOSUS* IN THE HYGIENIC
LABORATORY METHOD WITH BEEF INFUSION BROTH

Substance	Highest Germicidal Dilution		Phenol Coefficient	Mercuric Chlorid Coefficient
	2½ Minutes	15 Minutes		
Phenol.....	1:70	1:90		
Mercurophen No. 4.....	1:40,000	1:200,000	1396	+1.5
Mercurophen No. 6.....	1:40,000	1:160,000	1174	+1.4
Mercurophen No. 11.....	1:40,000	1:120,000	952	+1.1
Mercurophen No. 12.....	1:40,000	1:120,000	952	+1.1
Mercury benzoate.....	1:30,000	1:80,000	658	-0.78
Mercury zozoiiodolate.....	1:20,000	1:60,000	476	-0.56
Mercury cacodylate.....	1:10,000	1:30,000	238	-0.275
Mercuric chlorid.....	1:40,000	1:100,000	841	

TABLE 8

SUMMARY OF RESULTS OF GERMICIDAL TESTS WITH *B. TYPHOSUS* IN THE HYGIENIC
LABORATORY METHOD WITH BEEF EXTRACT BROTH

Substance	Highest Germicidal Dilution		Phenol Coefficient	Mercuric Chlorid Coefficient
	2½ Minutes	15 Minutes		
Phenol.....	1:70	1:90		
Mercurophen No. 4.....	1:40,000	1:160,000	1174	+1.5
Mercurophen No. 6.....	1:40,000	1:160,000	1174	+1.5
Mercurophen No. 11.....	1:40,000	1:120,000	952	+1.25
Mercurophen No. 12.....	1:40,000	1:120,000	952	+1.25
Mercury benzoate.....	1:30,000	1:80,000	658	-0.87
Mercury zozoiiodolate.....	1:20,000	1:60,000	476	-0.65
Mercury cacodylate.....	1:10,000	1:30,000	237	-0.31
Mercuric chlorid.....	1:40,000	1:80,000	729	

The results of a series of tests are shown in tables 5 and 6 and summarized in tables 7 and 8, the latter comparing the results observed with a beef infusion broth of +0.5 and reaction to those observed with neutral beef extract broth.

These results may be summarized as follows:

1. The phenol coefficient of mercurophen varied from 900 to 1,300 while that of mercuric chlorid was about 840.

³ Jour. Infect. Dis., 1911, 8, p. 1.

2. As shown in table 6 the phenol coefficient of mercurophen and the common mercurial compounds bore some relationship to the percentage of pure mercury. A mercurophen having 53% pure mercury showed a coefficient of 1,174 while a second showing on analysis about 47% mercury, had a coefficient of but 552. The results with mercury benzoate, mercury cacodylate and mercury zozoiolate all having varying percentages of mercury, show a similar bearing of mercury content on the results of these germicidal tests as was found with the antiseptic tests previously described.

3. As a general rule, mercurophen and mercuric chlorid killed *B. typhosus* in this test in a 1:400,000 dilution in $2\frac{1}{2}$ minutes, but in the longer period of exposure—namely, 15 minutes—mercurophen proved superior inasmuch as dilutions varying from 1:120,000 to 1:200,000 were germicidal while mercuric chlorid was germicidal in dilutions varying from 1:80,000 to 1:100,000; the higher phenol coefficients of mercurophen were largely due to this property of killing *B. typhosus* more quickly than mercuric chlorid and the other compounds.

This advantage of mercurophen is shown in the column of mercuric chlorid coefficients in tables 7 and 8, where mercuric chlorid is taken as 1.0 and the mercurophens and other compounds expressed accordingly. As previously stated in the antiseptic tests mercuric chlorid proved equal to or slightly superior to mercurophen; the differences are very probably to be ascribed to differences in the rapidity of action, mercurophen being somewhat more rapid in its typhoidicidal activity, as discussed below and shown in table 9.

Aside from this advantage mercurophen was more germicidal than expected inasmuch as it contains but 53% mercury as compared with 74% mercury in mercuric chlorid; this superiority is due to the peculiar constitution of the molecule of mercurophen, and constitutes an observation of great interest and importance from the viewpoint of chemotherapy.

4. As shown in tables 7 and 8 but very slight differences in the results were observed with beef infusion broth of +0.5 and neutral beef extract broth; at times the former medium showed slightly higher values as will be noted in the tables with mercurophen No. 4 and mercuric chlorid.

These results vary considerably from those previously reported with the Rideal-Walker method and we are at a loss to explain the discrepancies; mercurophen is not as germicidal by the Hygienic Labo-

ratory method as we originally believed. In the former work a strain of *B. typhosus* was employed which was isolated over 20 years ago and has been widely used in the agglutination tests; comparative tests with the Rawling's strain showed that the former grew more profusely in the same broth and is somewhat more difficult to kill but these differences do not explain all of the discrepancy. The phenol previously employed had been standardized about 2 months before the tests were made and inasmuch as phenol kills *B. typhosus* in dilution of 1:70 to 1:90 in 2½ to 15 minutes whereas our former phenol required 1:20 to 1:40 dilutions to kill the former strain of *B. typhosus*, it is likely that a large part of the error was due to defective stock solution of phenol; unfortunately none was available for further tests to settle this supposition in a more definite manner.

THE RAPIDITY OF GERMICIDAL ACTIVITY OF MERCUROPHEN AND OTHER MERCURIAL COMPOUNDS

Further evidence of the more rapid germicidal activity of mercurphen as compared with mercuric chlorid, is shown in the results of the following experiment:

In a series of test tubes were placed 10 cc of varying dilutions of the mercurial compounds in distilled water seeded with 0.1 cc of 24-hour broth cultures of the test micro-organism; the controls were prepared with sterile distilled water and culture alone. After intervals of 15, 30, 60 and 120 minutes at room temperature 0.1 cc of each tube was plated with 10 cc of agar at 45 C. and the plates counted after 48 hours' incubation; the results of one experiment shown in table 9 indicate that mercurphen is more rapidly germicidal for staphylococci and typhoid bacilli than equal strength dilutions of mercuric chlorid; for example, a 1:100,000 dilution of mercurphen was completely germicidal for staphylococci after an exposure of 1 hour and for typhoid bacilli in 15 minutes whereas mercuric chlorid failed to destroy all staphylococci in 2 hours and required 60 minutes for the complete destruction of the typhoid bacilli.

TABLE 9
THE RAPIDITY OF GERMICIDAL ACTIVITY OF MERCUROPHEN AND MERCURIC CHLORID

Substances	Dilution	Action on Staphylococci				Action on <i>B. typhosus</i>			
		15 Min.	30 Min.	60 Min.	120 Min.	15 Min.	30 Min.	60 Min.	120 Min.
Mercurphen.....	1:100,000	200	152	sterile	sterile	sterile	sterile	sterile	sterile
Mercuric chlorid.....	1:100,000	520	230	20	18	600	150	sterile	sterile
Mercurphen.....	1:500,000	unc.	unc.	unc.	2000	230	40	sterile	sterile
Mercuric chlorid.....	1:500,000	unc.	unc.	unc.	530	unc.	230	6	sterile
Mercurphen.....	1:1,000,000	unc.	unc.	unc.	unc.	unc.	unc.	unc.	unc.
Mercuric chlorid.....	1:1,000,000	unc.	unc.	unc.	unc.	unc.	unc.	unc.	unc.
Controls.....	0	unc.	unc.	unc.	unc.	unc.	unc.	unc.	unc.

The numbers give the colonies per plate; unc. = an uncountable number of colonies.

THE STABILITY OF SOLUTIONS OF MERCUROPHEN

Solutions of mercurophen in pure distilled water kept in tightly stoppered bottles to prevent evaporation do not undergo change in germicidal activity over long periods of time; the same was found true of solutions of mercuric chlorid kept under the same conditions (table 10). Exposure to light has no perceptible effect and solutions are easily kept at ordinary room temperature without any special precautions in regard to the container except the prevention of evaporation. The result of a single experiment bearing on the bacteriostatic activity of solutions of mercurophen and mercuric chlorid for the same strain of staphylococcus aureus over a period of 6 weeks, are shown in table 10. In this experiment both stock 1:1,000 solutions were kept in the laboratory near a window with abundant sunlight, in glass stoppered cylinders; the slight variations in bacteriostatic or antiseptic activity noted over this period of time are readily explained on the basis of experimental error and particularly to variations in the resistance of the strain of staphylococcus; the same culture medium was used throughout.

TABLE 10
THE STABILITY OF MERCUROPHEN AND MERCURIC CHLORID; HIGHEST ANTISEPTIC
DILUTION FOR STAPHYLOCOCCUS AUREUS

Age of Stock Solutions	Mercurophen	Mercuric Chlorid
Fresh.....	1:1,800,000	1:540,000
1 week.....	1:1,800,000	1:540,000
2 weeks.....	1:1,200,000	1:480,000
3 weeks.....	1:1,800,000	1:540,000
4 weeks.....	1:1,800,000	1:480,000
5 weeks.....	1:1,200,000	1:480,000
6 weeks.....	1:1,200,000	1:480,000

THE GERMICIDAL ACTIVITY OF MERCUROPHEN IN BLOOD,
ASCITES FLUID AND SERUM

In a menstruum rich in serum proteins the germicidal activity of mercurophen is reduced as compared with similar tests in which serum proteins are absent, but as stated in our preliminary report, the reduction in germicidal activity is not nearly so marked as that occurring with mercuric chlorid and other ordinary mercurial compounds, and with phenol; indeed, this is a distinguishing property of mercurophen as compared with most other mercurial compounds; a probable explanation lies in the fact that mercurophen is remarkably free of precipitating properties, as is shown in experiments summarized in table 13.

Germicidal Activity of Mercurophen in Blood.—In a series of experiments sterile oxalated rabbit and rat blood were employed; 20 cc of each were heavily infected with the addition of 2 cc of a 48-hour broth culture of *Staph. aureus* and incubated for an hour. In a series of small sterile test tubes equal parts of infected blood were mixed with solutions of mercurophen as 1:500 and 1:1,000, the final dilutions of mercurophen being 1:1,000 and 1:2,000 in a 50% oxalated blood, and allowed to stand at room temperature. At varying intervals 0.1 cc of each mixture was seeded in tubes of broth containing 10 cc or plated with 10 cc of agar at 45 C. and examined after 72 hours' incubation. The results are briefly summarized in tables 11 and 12.

TABLE 11
GERMICIDAL ACTIVITY OF MERCUROPHEN, MERCURIC CHLORID AND PHENOL IN
OXALATED BLOOD

Substance	Final Dilution in 50 per Cent. Blood	Exposure for Minutes					
		1	5	10	15	30	60
Mercurophen.....	1:1,000	—	—	—	—	—	—
Mercurophen.....	1:2,000	+	+	+	+	+	—
Mercuric chlorid.....	1:1,000	—	—	—	—	—	—
Mercuric chlorid.....	1:2,000	+	+	+	+	+	+
Phenol.....	1:40	+	+	+	—	—	—
Phenol.....	1:80	+	+	+	+	+	—
Salt solution control.....	+	+	+	+	+	+

— = sterile; + = growth.

TABLE 12
GERMICIDAL ACTIVITY OF MERCUROPHEN, MERCURIC CHLORID AND PHENOL IN
ASCITES FLUID

Substance	Final Dilution in 50 per Cent. Ascites Fluid	Exposure for Minutes					
		1	5	10	15	30	60
Mercurophen.....	1:2,000	—	—	—	—	—	—
Mercurophen.....	1:4,000	—	—	—	—	—	—
Mercuric chlorid.....	1:2,000	—	—	—	—	—	—
Mercuric chlorid.....	1:4,000	+	+	+	—	—	—
Phenol.....	1:80	—	—	—	—	—	—
Phenol.....	1:20	+	+	+	+	—	—
Salt solution control.....	+	+	+	+	+	+

— = sterile; + = growth.

As shown in table 11, a final dilution of 1:1,000 mercurophen in 50% blood was completely germicidal in 1 minute, whereas mercuric chlorid was not germicidal in 5 minutes; in 1:2,000 dilution mercurophen required 60 minutes to sterilize the blood, whereas mercuric chlorid failed in this period and indeed after 2 hours' exposure. Phenol in final dilution of 1:40 required 15 minutes, and in 1:80 required 60 minutes to bring about complete sterilization.

Hemolytic Activity of Mercurophen.—In this connection mention may be made of the results of experiments bearing on the hemolytic activity of solutions of mercurophen, mercuric chlorid and other mercurial compounds in

physiologic salt solution, for the washed erythrocytes of various mammals; mercurophen proved hemolytic for washed human erythrocytes in final dilutions 1:16,000 and mercuric chlorid as high as 1:160,000. For sheep cells mercurophen was hemolytic 1:80,000 and mercuric chlorid 1:160,000.

Germicidal Activity of Mercurophen in Ascites Fluid.—With 50% ascites fluid mercurophen in final dilution of 1:4,000 brought about complete sterilization within 1 minute, whereas mercuric chlorid failed in 10 minutes; phenol in final dilution of 1:120 required 30 minutes.

Germicidal Activity of Mercurophen in Serum.—These experiments were conducted at room temperature by mixing in sterile test tubes 2.5 cc of fresh, sterile unheated human serum with 2.5 cc of water solutions of the respective mercurial compounds and seeding the tubes with 0.1 cc of a paper filtered 24-hour broth culture of *Staph. aureus*. At varying intervals 0.1 cc of each mixture was plated in 10 cc of plain neutral agar at 45 C. and the plates counted at the end of 48 hours. The results shown in table 13 indicate the general superiority of mercurophen in a serum menstruum over other mercurial compounds; for example, mercurophen in final dilution of 1:10,000 proved germicidal after an exposure of 1 minute, whereas mercuric chlorid and the oxycyanid in equal dilutions required over 30 minutes to produce complete sterilization and the succinimid and cacodylate failed even after 2 hours in final dilution as low as 1:2,000.

TABLE 13

GERMICIDAL ACTIVITY OF MERCUROPHEN, MERCURIC CHLORID AND OTHER MERCURIAL COMPOUNDS IN 50% DILUTION OF SERUM FOR *STAPHYLOCOCCUS AUREUS*

Compound	Percent. Pure Mercury	Precipitation of Serum Proteins	Final Di- lution in 50% Serum	Results after Exposure for Minutes				
				1	15	30	60	120
Mercurophen.....	53	Neg. 1:200	1:50,000	3500	1000	410	71	10
Mercurophen.....	53	Neg. 1:200	1:10,000	sterile	sterile	sterile	sterile	sterile
Mercuric chlorid.....	74	Pos. 1:1,000	1:50,000	unc.	unc.	unc.	unc.	unc.
Mercuric chlorid.....	74	Pos. 1:1,000	1:10,000	unc.	1990	100	sterile	sterile
Hg oxycyanid.....	83	Pos. 1:400	1:50,000	unc.	unc.	unc.	unc.	unc.
Hg oxycyanid.....	83	Pos. 1:400	1:10,000	unc.	1700	300	sterile	sterile
Hg succinimid.....	50	Neg. 1:200	1:50,000	unc.	unc.	unc.	unc.	unc.
Hg succinimid.....	50	Neg. 1:200	1:2,000	unc.	unc.	unc.	unc.	unc.
Hg cacodylate.....	20	Pos. 1:300	1:2,000	unc.	unc.	unc.	unc.	unc.
Control.....	0.	0	unc.	0	0	0	unc.

Furthermore, the precipitation or coagulation of serum proteins by mercurial compounds does not necessarily parallel the degree of inhibition of germicidal activity of these substances in serum. For example, mercurophen and mercury succinimid are organic compounds containing 53 and 50% pure mercury, respectively, and neither precipitates protein in dilutions as low as 1:200, but the succinimid is over 25 times less germicidal in serum; mercuric chlorid precipitates serum proteins in final dilution as high as 1:1,000 with equal parts of serum, but is equal in germicidal activity to the oxycyanid which precipitates protein only in final dilutions as low as 1:400.

While the technic of these tests does not permit comparison with the results of our former tests conducted after the pipet method of Wright, the general conclusion remains unaltered regarding the superior germicidal activity of mercurophen in a medium rich in proteins as uncoagulated blood, ascites fluid and serum.

THE INFLUENCE OF MERCUROPHEN ON SERUM PROTEINS

Solutions of mercurophen are practically devoid of all precipitating influence on serums, and in view of its germicidal activity this property is highly desirable. With hot water a 1:100 solution of mercurophen may be prepared and a mixture of equal parts of this solution and fresh human serum may show slight clouding but no precipitation, even after standing 24 hours; higher dilutions have no visible effect at all on serum.

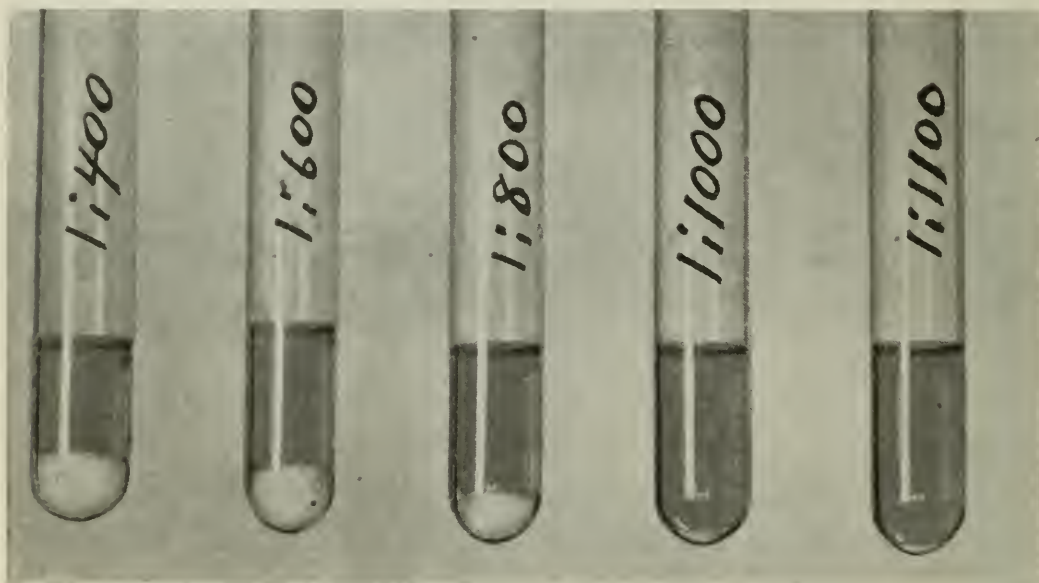


Fig. 1.—The precipitation of serum proteins by mercuric chlorid.

Studies made with other mercurial compounds have shown that this precipitation or coagulation of serum proteins bears no relation to the content in mercury but depends on the nature of the compound, that is, whether organic or inorganic, and the strength of the acid from which a particular salt is derived. For example, mercuric chlorid and mercury sulphate are inorganic compounds and precipitate serum more actively and in higher dilutions than such mercury salts of organic acids as the acetate, oxycyanid and cacodylate of mercury (table 14), although both of the inorganic compounds contain less mercury than one of the salts of an organic acid, namely, the oxycyanid. Furthermore, mercuric chlorid containing 74% mercury produces less precipitate than the sulphate containing 67% and of the salts of organic acids, the oxycyanid containing 83% mercury is hardly more active in precipitating proteins than the cacodylate with but 20% mercury.

Of the two compounds studied only two, namely, mercurophen and mercury succinimid, were found without any effect on serum in dilutions as low as 1:200, and in view of its high germicidal activity the former is rendered peculiarly valuable for the practical disinfection of substances relatively rich in precipitable protein (Figs. 1 and 2).

TABLE 14

THE PRECIPITATION OF HUMAN SERUM PROTEINS BY MERCUROPHEN, MERCURIC CHLORID AND OTHER MERCURIAL COMPOUNDS

Substances	Nature of Compound	Percent- age of Pure Hg	Final Dilutions							
			1:200	1:300	1:400	1:600	1:800	1:1,000	1:1,200	1:1,600
Hg bichlorid.....	Inorganic salt	74	4+	4+	4+	4+	2+	+	—	—
Hg sulphate.....	Inorganic salt	67	+	+	+	+	+	+	—	—
Hg acetate.....	Organic salt	63	3+	+	—	—	—	—	—	—
Hg oxycyanid.....	Organic salt	83	3+	+	+	—	—	—	—	—
Hg cacodylate.....	Organic salt	20	2+	—	—	—	—	—	—	—
Hg succinimid.....	Pseudo-complex organic salt	50	—	—	—	—	—	—	—	—
Mercuriophen.....	Half-complex organic salt	53	—	—	—	—	—	—	—	—

— = no precipitate; 4+ = very heavy precipitate after standing 24 hours.

In preparing a 1:100 solution of mercury sulphate it was necessary to add 1% sulphuric acid to the water; equal parts of this diluted acid and serum produced no precipitation. All tubes containing dilutions of mercury sulphate showed marked clouding with but slight amounts of precipitate, due probably to solution of the precipitate albumin in the acid fluid.



Fig. 2.—The nonprecipitable influence of mercuriophen on serum proteins.

RESULTS OF TESTS BEARING ON THE PRACTICAL DISINFECTING PROPERTIES OF MERCUROPHEN

Although mercurial compounds as a group have largely fallen into disuse in favor of formalin and derivatives of coal tar, despite the high germicidal activity of many of them as disinfectants for the skin and such discharges as urine, feces and sputum and the sterilization of instruments, we have extended our studies with mercuriophen in a series of experiments to determine its practical disinfecting power.

particularly in view of the fact that it does not precipitate protein and maintains a high degree of germicidal activity in the presence of a rich protein medium.

Disinfection of Urine.—Tests have been conducted with urine containing typhoid bacilli alone and with urine as ordinarily voided and collected and containing large numbers of various bacteria. In conducting the experiments equal parts of urine and mercurophen solution were mixed, allowed to stand at laboratory temperature and 0.1 cc plated in 10 cc of agar at 45 C. at varying intervals, the plates being examined after 72 hours' incubation. Mercuric chlorid, phenol, and salt solution controls were included in each experiment.

The results may be expressed as follows:

1. With typhoid urine of weakly acid reaction and albumin free, mercurophen always produced complete sterilization within 5 minutes in final dilution as high as 1:8,000; mercuric chlorid required about 30 minutes in a similar dilution and phenol about 15 minutes in dilution of 1:80 (table 15).

TABLE 15
GERMICIDAL ACTIVITY OF MERCUROPHEN, MERCURIC CHLORID AND PHENOL FOR
B. TYPHOSUS IN URINE

Substance	Final Dilutions	Results after Exposure for Minutes					
		5	10	15	30	45	60
Mercurophen.....	1:4 000	—	—	—	—	—	—
Mercurophen.....	1:8,000	—	—	—	—	—	—
Mercuric chlorid.....	1:4,000	—	—	—	—	—	—
Mercuric chlorid.....	1:8 000	+	+	+	—	—	—
Phenol.....	1:80	+	+	—	—	—	—
Salt solution control.....	+	+	+	+	+	+

— = sterile; + = growth.

2. With ordinary urine of neutral or weakly acid reaction, albumin free but clouded with various bacteria, mercurophen in final dilution of 1:2,000 usually produced complete sterilization within 5 minutes while a 1:4,000 dilution required an hour; mercuric chlorid in final dilution of 1:2,000 required an hour while a 1:4,000 dilution failed. Phenol in final dilution of 1:80 required about 45 minutes (table 16).

TABLE 16
GERMICIDAL ACTIVITY OF MERCUROPHEN, MERCURIC CHLORID AND PHENOL FOR THE
BACTERIA PRESENT IN URINE AS ORDINARILY COLLECTED

Substance	Final Dilutions	Results after Exposure for Minutes					
		5	10	15	30	45	60
Mercurophen.....	1:2,000	—	—	—	—	—	—
Mercurophen.....	1:4,000	+	+	+	+	+	—
Mercuric chlorid.....	1:2,000	+	+	+	+	+	—
Mercuric chlorid.....	1:4,000	+	+	+	+	+	+
Phenol.....	1:80	+	+	+	+	—	—
Salt solution control.....	...	+	+	+	+	+	+

— = sterile; + = growth.

Most interest concerns the disinfection of the urine of persons suffering with typhoid fever, and mercurphen in final dilution of 1:5,000 secured by mixing 1 part of a 1:1,000 solution of mercurphen with 4 parts of urine may be relied on to accomplish this purpose with an exposure of at least 5 minutes at ordinary room temperature.

Disinfection of Feces.—As stated in our previous communication, mercurphen in final dilution of 1:5,000 with an emulsion of feces made by shaking 1 gm. in 100 cc of sterile distilled water, produced complete sterilization at room temperature in a period of 30 minutes or less; with mercuric chlorid a dilution of 1:2,000 was required for similar results.

Inasmuch as this emulsion of feces was more dilute than usually dealt with in actual practice we have conducted a series of experiments consisting in adding to fresh semi-solid feces an equal weight of different solutions of mercurphen, mercuric chlorid and phenol; as a general rule an equal weight of the disinfectant was in volume closely parallel by rough measurement, to the volume of feces.

After the addition of the disinfectant solution to the feces the material was thoroughly broken up, but it was not always possible to avoid numerous clumps of feces large enough to protect bacteria within them. After standing at room temperature for varying intervals of time, one 4 mm. loopful of each mixture was plated with 10 cc of agar at 45 C. and the plates counted after 48 hours' incubation.

The results were irregular depending largely on the consistency of the feces and the thoroughness of mixture with the disinfectants. With diarrheal stools and those secured after brisk catharsis with salines, the results were more regular and the material more easily sterilized than the case with the normal semi-solid feces.

TABLE 17
THE DISINFECTION OF FECES BY MERCUROPHEN, MERCURIC CHLORID AND PHENOL

Substances	Final Dilution	Results after Exposure for Minutes				
		10	20	30	45	60
Mercurphen.....	1:1,000	—	—	—	—	—
Mercurphen.....	1:2,000	unc.	380	290	150	—
Mercuric chlorid.....	1:1,000	few	—	—	—	—
Mercuric chlorid.....	1:2,000	unc.	unc.	unc.	420	140
Phenol.....	1:40	unc.	unc.	400	150	80

— = sterile; unc. = an uncountable number of colonies.

The results of one experiment with semi-solid feces are shown in table 17; in this experiment equal weights of feces and 1:500 and 1:1,000 solutions of mercurphen and mercuric chlorid and 1:20 phenol were mixed and plated at intervals varying from 10-60 minutes.

The results are fairly representative of the series of experiments and show that a final dilution of mercurophen 1:1,000 produces sterilization within 10 minutes, while a 1:2,000 dilution requires at least 1 hour (table 17); similar results were observed with mercuric chlorid although inferior to mercurophen, whereas phenol in 1:40 failed to produce complete sterilization within the hour.

Examination of the plates usually showed a predominating number of spore-forming bacteria; gram-negative bacilli of the typhoid-colon group were generally absent, and we feel quite certain that the addition to the semi-solid typhoid stool of an equal volume of 1:1,000 solution of mercurophen will result in the complete destruction of typhoid bacilli within a period of 1 hour at room temepature.

Disinfection of Sputum.—Experiments on the disinfection of sputum consisted in thoroughly mixing equal parts of sputum with varying dilutions of the disinfectant and plating 0.1 cc of each mixture with 10 cc of agar at 45 C. after varying intervals of exposure at room temperature.

The results of one experiment conducted with a thin sanguineous sputum of pneumonia complicating influenza and containing pneumococci, nonhemolytic streptococci and other organisms is shown in table 18; the results of this and similar experiments have shown that the mixture of equal parts of sputum and 1:2,000 mercurophen (final 1:4,000 dilution) results in sterilization within a few minutes, whereas mercuric chlorid failed to sterilize in this dilution in periods of 30 minutes and required 15 minutes to produce sterilization with a 1:2,000 dilution.

TABLE 18
THE DISINFECTION OF SPUTUM BY MERCUROPHEN, MERCURIC CHLORID AND, PHENOL

Substance	Final Dilutions	Results after Exposure for Minutes					
		1	3	5	10	15	30
Mercurophen.....	1:2,000	—	—	—	—	—	—
Mercurophen.....	1:4,000	—	—	—	—	—	—
Mercuric chlorid.....	1:2,000	+	+	+	+	—	—
Mercuric chlorid.....	1:4,000	+	+	+	+	+	+
Phenol.....	1:40	+	+	+	+	+	—
Salt solution control.....	+	+	+	+	+	+

— = sterile; + = growth.

With thick gelatinous sputa of lobar pneumonia and the caseous sputum of tuberculous persons, stronger solutions of mercurophen were required for sterilization, but as a general rule the addition of an equal part of 1:1,000 solution of mercurophen to such sputa resulted in complete sterilization within an hour except in the case of

large caseous or gelatinous masses of sputum which resisted for that time permeation of the disinfectant.

The Disinfection of Catheters.—Experiments on the sterilization of rubber catheters were conducted with ordinary rubber tubing cut into pieces of 1 inch length, soaked for an hour in a broth culture of *Staph. aureus* and dried in the air. A number of such pieces of tubing were placed in beakers containing 1:5,000 solution of mercurophen and mercuric chlorid and 1:40 phenol and kept at room temperature; after varying intervals of exposure pieces of tubing were removed from the several disinfectants, washed with sterile water and planted in flasks of glucose broth. Controls were conducted in the same manner with normal salt solution. After 48 hours' incubation the results were recorded and those shown in table 19 are representative of the results observed in a series of tests.

TABLE 19

RESULTS OF DISINFECTION TESTS EMPLOYING INFECTED RUBBER TUBING AND SOLUTIONS OF MERCUROPHEN, MERCURIC CHLORID AND PHENOL

Substance	Dilution	Exposure for Minutes						
		1	3	5	8	10	15	20
Phenol.....	1:40	+	+	+	+	+	+	+
Mercuric chlorid.....	1:5,000	+	+	+	+	—	—	—
Mercurophen.....	1:5,000	+	+	+	—	—	—	—
Salt solution control.....	—	+	+	+	+	+	+	+

+ = growth; — = sterile.

As shown in table 19 mercurophen in dilution of 1:5,000 usually produced complete sterilization with an exposure of 8 minutes, whereas mercuric chlorid required 10 minutes or more and phenol in 1:40 dilution failed to sterilize even after 20 minutes' exposure. With tubing heavily infected with spore-bearing bacilli like *B. subtilis*, much stronger dilutions of both mercurophen and mercuric chlorid were required as 1:500 solutions with periods of exposure approximating 30 minutes.

The results of these experiments have shown that catheters which cannot be readily sterilized with heat, may be sterilized with a 1:5,000 dilution of mercurophen in an exposure of at least 10 minutes.

Disinfection of Rubber Gloves.—Experiments conducted with surgeons' gloves contaminated with staphylococci have shown that immersion in a warm solution of mercurophen 1:5,000 results in sterilization within a period of 1 minute exposure, whereas mercuric chlorid requires an exposure of 1-3 minutes; in 1:2,000 dilution both mercurophen and mercuric chlorid bring about sterilization within a minute.

Disinfection of Instruments.—Experiments on the disinfection of metal instruments were conducted by contaminating scissor blades, scalpels and hemostatic forceps by immersion in broth cultures of *Staph. aureus* followed by drying in the air. These instruments were then placed in beakers contain-

iny varying dilutions of the disinfectant, and normal salt solution as a control and after varying intervals of exposure at room temperature was removed with sterile forceps, washed with sterile water to remove disinfectant and cultured by placing each in a large flask of broth. The results observed with one experiment are shown in table 20; the controls invariably showed heavy growths.

TABLE 20
THE DISINFECTION OF INSTRUMENTS BY MERCUROPHEN, MERCURIC CHLORID AND PHENOL

Substance	Final Dilution	Duration of Exposure for Minutes			
		1	5	10	15
Mercurophen.....	1:5,000	—	—	—	—
Mercurophen.....	1:10,000	+	—	—	—
Mercuric chlorid.....	1:5,000	—	—	—	—
Mercuric chlorid.....	1:10,000	+	+	+	+
Phenol.....	1:40	+	+	+	+

— = sterile; + = growth.

Mercurophen and mercuric chlorid in dilution of 1:5,000 usually disinfected such instruments after an exposure of 1 minute; in 1:10,000 dilution mercurophen required from 1-5 minutes while mercuric chlorid in equal strength usually failed to disinfect in a period of 15 minutes. Phenol in dilution of 1:40 likewise failed to disinfect these instruments within the 15 minute period of exposure.

In a second series of experiments instruments were picked up without previous preparation, dropped into flasks containing equal parts of nutrient broth and disinfectant solution and incubated for 48-72 hours. With this technic mercurophen in final dilution as high as 1:15,000 proved antiseptic, while mercuric chlorid was antiseptic in 1:3,000 and phenol in 1:40 to 1:60 (table 21).

TABLE 21
THE DISINFECTION OF INSTRUMENTS BY MERCUROPHEN, MERCURIC CHLORID AND PHENOL

Substance	Final Dilutions and Results							
	1: 2,000	1: 4,000	1: 6,000	1: 8,000	1: 10,000	1: 12,000	1: 15,000	1: 18,000
Mercurophen.....	—	—	—	—	—	—	—	+
Mercuric chlorid.....	—	—	—	—	+	+	+	+
Phenol.....	1:40	1:80	1:100	1:120	0	0	0	0

— = sterile; + = growth; controls showed heavy growths.

These experiments have shown that mercurophen in 1:5,000 dilution and even as high as 1:10,000 can be relied on for the sterilization of such instruments as cannot be sterilized with heat, with an exposure of at least 5 minutes; furthermore, the objectionable deleterious influence of mercuric chlorid on nickel plated and steel instruments with tarnishing, is not true of mercurophen inasmuch as the latter substance does not tarnish metals (Fig. 3).

THE NONTARNISHING EFFECT OF MERCUROPHEN
ON INSTRUMENTS

Experiments concerning the influence of mercurophen and other mercurial compounds on surgical instruments have been conducted by immersing steel, nickel and silver-plated instruments in 1:1,000 solu-

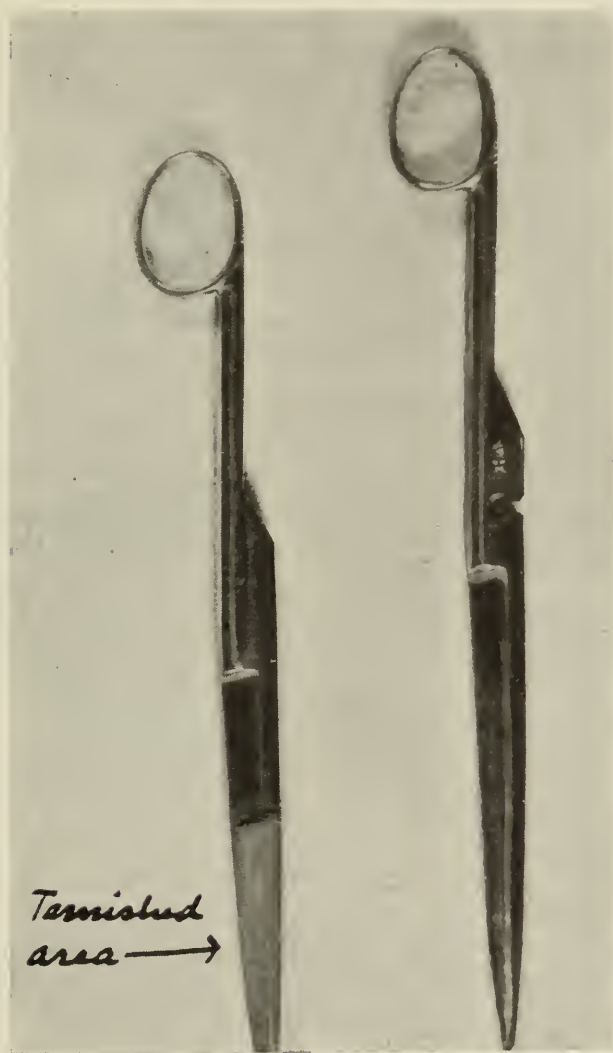


Fig. 3.—The tarnishing of a nickel-plated instrument (left) by mercuric chlorid in dilution of 1:1,000; absence of tarnishing of similar instrument by mercurophen in dilution of 1:1,000 (right).

tions for periods of 24-48 hours at room temperature. Of the mercurial compounds tested in this manner only mercurophen and mercury succinimid did not tarnish any of these instruments to the slightest degree, whereas mercuric chlorid, mercury acetate, mercury sulphate, mercury cacodylate and mercury oxycyanid produced considerable

tarnishing particularly of steel but not of silver-plated instruments (Fig. 3); as is well known, mercuric chlorid cannot be used for the disinfection of surgical instruments by reason of this deleterious influence, as this compound not only tarnishes steel and nickel-plated instruments but may even discolor silver-plated instruments on prolonged exposure.

The question of tarnishing does not depend on the percentage of mercury in a compound (table 22), but rather on the ease with which mercury is precipitated out of solution by nickel and steel; for example, mercuric chlorid, mercury acetate and mercury sulphate are compounds in which mercury exists in a solution as a free ion and these compounds tarnish metals and are most likely to precipitate proteins. Of the compounds tested, only mercurophen and mercury succinimid were found free of this tarnishing effect on instruments and either may be used with impunity for the disinfection of instruments in as low dilution as 1:1,000.

TABLE 22
THE INFLUENCE OF 1:1,000 SOLUTIONS OF MERCUROPHEN AND OTHER MERCURIAL COMPOUNDS ON THE METAL OF SURGICAL INSTRUMENTS

Substance	Percentage Pure Hg	Nature of Compound	Results	
			Silver Plated	Steel and Nickel Plated
Mercurophen.....	53	Half complex organic salt	—	—
Hg succinimid.....	50	Pseudocomplex organic salt	—	—
Hg oxycanid.....	83	Organic salt	—	+
Hg cacodylate.....	20	Organic salt	—	+
Hg acetate.....	63	Organic salt	—	+
Hg sulphate.....	67	Inorganic salt	—	+
Mercuric chlorid.....	74	Inorganic salt	+	+

Results after 48 hours exposure at room temperature; — = no effect; + = tarnishing.

Disinfection of the Skin.—In these experiments both hands of a series of laboratory assistants were cultured without previous washing, by rigorous rubbing of the skin in various parts with sterile swabs moistened with sterile broth or removal of epidermis with sterile scalpels followed by inoculation of tubes of glucose broth and 48 hours' incubation. As expected, all of these cultures showed heavy growths of various bacteria in which staphylococci and *B. subtilis* predominated. Immediately after the preliminary cultures were made the left hand was completely immersed in a sterile basin containing the disinfectant solution at 40 C. and the right hand in a similar basin containing sterile normal salt solution at the same temperature as a control.

After varying intervals of exposure to disinfectant the left hand was flushed with large volumes of sterile water at 40 C. to remove the disinfectant solution and both hands again cultured with particular attention to the finger nails and the skin between the fingers. The right or control hand invariably showed

growths after the longest period of immersion in the warm sterile salt solution; a summary of the results with mercurophen and mercuric chlorid in these experiments is given in table 23.

TABLE 23

RESULTS OF HAND DISINFECTION TESTS WITH MERCUROPHEN AND MERCURIC CHLORID

Substance	Dilution	Exposure and Results								
		Pre-lim.	1 Min.	3 Min.	5 Min.	7 Min.	10 Min.	12 Min.	15 Min.	25 Min.
Mercurophen No. 6.....	1:1,000	+	—	—	—	—	—	—	—	—
Mercurophen No. 6.....	1:5,000	+	+	—	—	—	—	—	—	—
Mercurophen No. 6.....	1:10,000	+	+	+	+	+	—	—	—	—
Mercurophen No. 6.....	1:40,000	+	+	+	+	+	+	+	—	—
Mercuric chlorid.....	1:1,000	+	+	+	—	—	—	—	—	—
Mercuric chlorid.....	1:5,000	+	+	+	+	—	—	—	—	—
Mercuric chlorid.....	1:10,000	+	+	+	+	+	+	+	+	—
Mercuric chlorid.....	1:40,000	+	+	+	+	+	+	+	+	+

+ = growth; — = sterile.

In some experiments cultures made of the hands after immersion in 1:40,000 mercurophen for this period of time, showed growth of bacteria.

As a general rule, a warm solution of mercurophen in strength of 1:1,000 disinfected the skin within 1 minute and 1:5,000 disinfected in from 1-3 minutes and mercuric chlorid in the same strength in from 5-7 minutes; in 1:10,000 dilution mercurophen required at least 10 minutes, and mercuric chlorid almost half an hour. In higher dilutions the results were more irregular; for example, mercurophen in dilution of 1:40,000 sometimes disinfected the skin after an exposure of 15 minutes or more, depending apparently on the condition of the skin, inasmuch as this factor was quite variable because the skin was not previously prepared. Mercuric chlorid invariably failed to disinfect the skin in these higher dilutions over a 30-minute period of exposure.

Similar experiments with the skin of hands after preliminary washing and scrubbing with a soft brush, green soap and hot water for a period of 5 minutes followed by rinsing in hot sterile water, yielded more irregular results inasmuch as the control hands were occasionally sterile and the action of mercurophen and mercuric chlorid more effective in higher dilutions or with shorter exposures. For use in operating rooms a hot solution of 1:5,000 mercurophen with a period of immersion of at least 1 minute following the usual preliminary cleaning, can be recommended on the basis of these experiments.

The Nonirritant Properties of Mercurophen on the Skin.—In this connection emphasis may be placed on several observations indicating that solutions of mercurophen do not produce the dermatitis with which some persons suffer following the use of the ordinary mercurial compounds and particularly mercuric chlorid. Three physicians who suffer with a mercurial dermatitis following contact with solutions of mercuric chlorid, have voluntarily acquainted us with these observations and are able to use mercurophen in dilutions as low as 1:2,000 with impunity.

On the Eye.—In order to test the comparative irritant properties of mercurophen, particularly in view of the findings of several ophthalmologists indicating that mercurophen has remarkable curative properties in pneumococcus conjunctivitis, solutions of different strength have been dropped into the eyes of rabbits. These experiments were conducted by daily instillation of mercurophen solution into the right eye and an equal amount of a similar solution of

mercuric chlorid into the left eye of each animal as a control. With 1:100 solutions of mercuric chlorid a severe purulent conjunctivitis was produced after 1 or 2 instillations, whereas after 3 and 4 instillations with 1:100 mercurophen but slight congestion was produced (Figs. 4 and 5); with 1:1,000 solutions, mercurophen produced no visible effects after 7 successive instillations, whereas mercuric chlorid produced slight conjunctivitis with erythema and slight discharge.

Solutions of mercurophen in strength of 1:1,000 and higher have also been used in the treatment of infantile gonococcus vaginitis by daily instillation into the vagina, with no evidences of irritation of the vaginal mucosa and introitus.

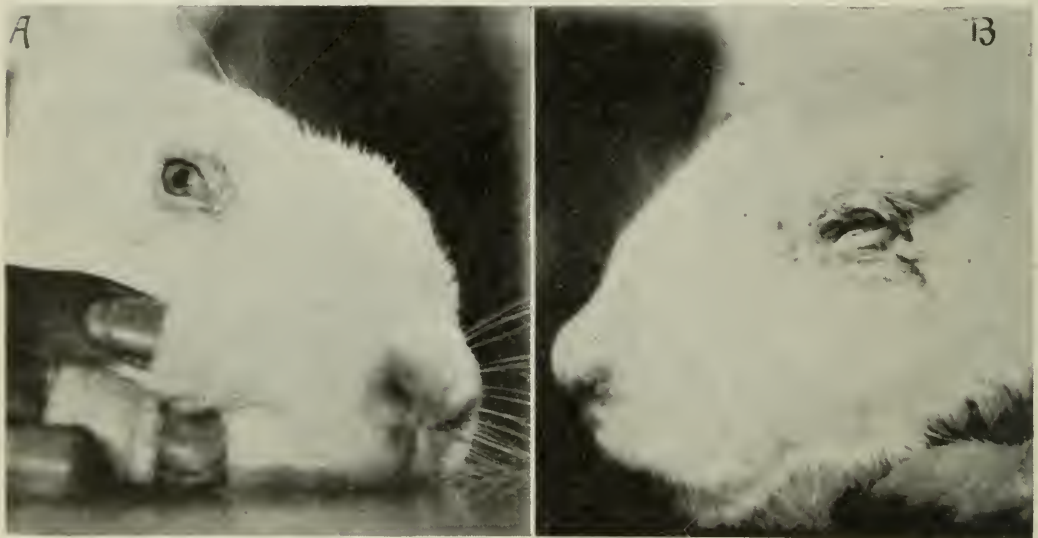


Fig. 4.—A, Eye of rabbit after three instillations of 2 drops of mercurophen in dilution of 1:100; mild hyperemia of conjunctiva.

B, Eye of rabbit after two instillations of 2 drops of mercuric chlorid in dilution of 1:100; severe purulent conjunctivitis.

These experiences have shown that mercurophen is remarkably free of irritant effects and that it may be used in contact with the skin and mucous membranes in dilutions as concentrated as 1:1,000 without apparent harm.

The Disinfection of Pus.—The results of these experiments varied according to the consistency of the pus, degree of phagocytosis and the type of infecting micro-organisms. In one experiment pus from an empyema which proved sterile was contaminated by adding 1 cc of a 24-hour broth culture of *Staph. aureus* to each 9 cc of pus and incubating several hours. Cultures of this material yielded heavy growths of staphylococci. Equal parts of this pus and varying dilutions of the disinfectants were well mixed, kept at room temperature and subcultured at intervals by transferring a 4 mm. loopful to glucose agar. The results of this experiment are shown in table 24; mercurophen in final dilution of 1:200 sterilized the pus in 5 minutes and in 1:5,000 dilution in 30 minutes. Mercuric chlorid in final dilution of 1:2,000 required 15 minutes and in 1:5,000 dilution failed in the longest period of exposure, namely,

30 minutes; phenol in final dilution of 1:40 required 5 minutes and in 1:80 required 30 minutes for complete disinfection.

TABLE 24
GERMICIDAL ACTIVITY OF MERCUROPHEN, MERCURIC CHLORID AND PHENOL IN STAPHYLOCOCCUS INFECTED PUS

Substance	Final Dilution	Results after Exposure for Minutes				
		1	5	10	15	30
Mercurphen.....	1:1,000	—	—	—	—	—
Mercurphen.....	1:2,000	+	—	—	—	—
Mercurphen.....	1:5,000	+	+	+	+	—
Mercuric chlorid.....	1:1,000	—	—	—	—	—
Mercuric chlorid.....	1:2,000	+	+	+	—	—
Mercuric chlorid.....	1:5,000	+	+	+	+	+
Phenol.....	1:40	+	—	—	—	—
Phenol.....	1:80	+	+	+	+	—
Control.....	+	+	+	+	+

— = sterile; + = growth.

In a second experiment employing pus of an empyema containing large numbers of a virulent streptococcus with some phagocytosis, 0.5 cc of pus was placed in each of a series of large sterile test tubes with 0.5 cc of varying dilutions of disinfectant; after standing for 15 minutes at room temperature 10 cc of serum glucose agar at 45 C. was added to each tube, mixed and poured in sterile petri dishes following by incubation for 4 days. The results showed complete sterilization by mercurphen in final dilution as high as 1:12,000 and mercuric chlorid in dilutions to 1:3,200; phenol was not employed.

In this connection it may be stated that experiments with varying dilutions of mercurphen and other mercurial compounds with emulsions of guinea-pig spleen, kidney and liver, have shown the entire absence of solvent action such as occurs with Dakin's solution and allied chlorin compounds.

TABLE 25
THE GERMICIDAL ACTIVITY OF MERCUROPHENIZED GAUZE FOR STAPHYLOCOCCUS AUREUS

Substance	Gauze Impregnated with Dilution	Results after Exposure for Minutes			
		1	5	15	30
Mercurphen.....	1:1,000	—	—	—	—
Mercurphen.....	1:5,000	—	—	—	—
Mercurphen.....	1:10,000	+	+	—	—
Mercuric chlorid.....	1:1,000	—	—	—	—
Mercuric chlorid.....	1:5,000	+	+	+	—
Mercuric chlorid.....	1:10,000	+	+	+	+

— = sterile; + = growth.

The Germicidal Activity of Mercurphenized Gauze.—Ordinary surgical gauze was thoroughly saturated with 1:1,000, 1:5,000 and 1:10,000 solutions of mercurphen and similar dilutions of mercuric chlorid and dried at 50 C. Small squares measuring about 1 inch and of 4 thicknesses were placed for 1 minute in a 24-hour broth culture of Staph. aureus, the excess of broth removed by gentle squeezing and each portion so treated placed in separate sterile petri dishes. At varying intervals these squares of gauze were planted in flasks containing 500 cc of nutrient broth in order to highly dilute the disinfectant car-

ried over, and incubated for 4 days. The results of an experiment are shown in table 25; gauze impregnated with a 1:5,000 dilution of mercuriofen proved completely germicidal after 1 minute of contact with the cocci and in 1:10,000 dilution proved germicidal within 15 minutes. Gauze impregnated with a 1:5,000 solution of mercuric chlorid required about 30 minutes of contact to destroy all of the cocci and the 1:10,000 gauze failed to sterilize in the 30-minute period of exposure.

Similar experiments with pus yielded irregular results according to the density of the pus, the degree of phagocytosis and the thickness of the layer of pus in contact with the gauze. The experiments have shown, however, that gauze impregnated with a 1:500 solution of mercuriofen is strongly germicidal and likely to prove useful in the dressing of wounds when a nonirritant disinfectant will aid in reducing bacterial activity.

THE TOXICITY OF MERCUIOPHEN

The toxicity of mercuriofen has been studied by determining the highest tolerated dose for white rats by intravenous injection and for white mice by intraperitoneal injection, over a period of 10 days.

TABLE 26
RESULTS OF TOXICITY TESTS WITH MERCUIOPHEN; INTRAVENOUS INJECTION INTO RATS

Weight in Grams	Dose per Kilo	Amount of 1:1,000 Solution Injected in C C	Time of Injection in Seconds	Results in Days						
				1	2	3	4	5	6	10
235	0.02	0.7 (1:100)	84	D						
220	0.01	2.2	264	—	—	—	—	—	—	D
95	0.009	0.9	106	—	—	D	—	—	—	—
90	0.008	0.8	96	—	—	—	—	—	—	—
95	0.007	0.7	84	—	—	D	—	—	—	—
165	0.006	1.0	120	—	—	—	—	—	—	—
130	0.005	0.65	78	—	—	—	—	—	—	—
85	0.004	0.3	36	—	—	—	—	—	—	—

In this experiment the mercuriofen contained 53% of mercury.

TABLE 27
RESULTS OF TOXICITY TESTS WITH MERCURIC CHLORID; INTRAVENOUS INJECTION INTO RATS

Weight in Grams	Dose per Kilo	Amount of 1:1,000 Solution Injected in C C	Time of Injection in Seconds	Results in Days						
				1	2	3	4	5	6	10
105	0.010	1.1	132	D						
120	0.008	0.96	120	—	D					
110	0.007	0.77	92	—	D					
120	0.006	0.72	86	—	—	D				
150	0.005	0.75	90	—	—	—	D			
150	0.004	0.6	72	—	—	—	—	—	—	D
150	0.003	0.45	54	—	—	—	—	—	—	—
100	0.002	0.2	60	—	—	—	—	—	—	—

The mercuric chlorid contained 74% mercury.

Dosis Tolerata by Intravenous Injection.—In these experiments white rats weighing from 100-200 gm. were employed; each animal was weighed and injected with 1:1,000 solutions in water in doses varying from 0.002-0.02 gm. per 1000 gm. of weight. The injections were made into the saphenous vein by gravity and at the rate of about 0.5 cc of solution per minute. All animals were kept under observation for a period of 10 days.

For comparative results other compounds containing varying amounts of mercury, as mercuric chlorid, mercury succinimid and mercury cacodylate were injected into rats in the same manner.

The results of several experiments are shown in tables 26, 27, 28, 29 and 30; as is the general rule in such experiments irregular deaths have occurred rendering multiple tests necessary in order to arrive at general conclusions.

TABLE 28

RESULTS OF TOXICITY TESTS WITH MERCURY CACODYLATE; INTRAVENOUS INJECTION INTO RATS

Weight in Grams	Dose per Kilo	Amount of 1:1,000 Solution Injected in C C	Time of Injection in Seconds	Results in Days					
				1	2	3	4	5	6 10
100	0.02	2.0	240	—	—	—	D		
115	0.01	1.6	192	—	—	—	—	D	
105	0.009	0.95	120	—	—	D			
120	0.008	0.96	120	—	—	—	—	—	—
105	0.007	0.74	89	—	—	—	—	—	—
85	0.006	0.51	62	—	—	—	—	—	—

The cacodylate contained 20% mercury.

TABLE 29

RESULTS OF TOXICITY WITH MERCURY SUCCINIMID; INTRAVENOUS INJECTION INTO RATS

Weight in Grams	Dose per Kilo	Amount of 1:1,000 Solution Injected in C C	Time of Injection in Seconds	Results in Days					
				1	2	3	4	5	6 10
145	0.01	1.45	174	—	—	—	D		
175	0.008	1.4	168	—	—	D			
170	0.007	1.2	144	—	—	—	D		
120	0.006	0.7	84	—	—	—	—	—	—
80	0.005	0.4	48	—	—	D			
95	0.004	0.38	45	—	—	—	—	—	—
90	0.002	0.18	24	—	—	—	—	—	—

The succinimid contained 50% mercury.

With the method of injection employed, namely, slow administration from a graduated pipet by gravity with a very satisfactory apparatus designed by Dr. G. C. Lake of the Hygienic Laboratory of the U. S. Public Health Service for testing the toxicity of arsphenamin, the highest tolerated dose of mercuriofen was about 0.008 gm. per kg. of body weight; mercuric chlorid was about 0.003-0.004 gm.; the succinimid of mercury about 0.006 gm. and the cacodylate of mercury about 0.008 gm. per kilogram of weight.

In our former studies on the comparative toxicity of mercurial compounds,⁴ the intravenous injections into rats were made with a syringe into the jugular vein, the injection being quite rapid and not infrequently made in a few seconds, the dose per rat being dissolved in exactly 1 cc of water. With this technic the dosis tolerata of mercuric chlorid was from 0.002-0.004 gm. per kg. of body weight, usually being nearer the 0.002 gm. dose; the dosis tolerata of the succinimid was about 0.002 gm. and the cacodylate of mercury averaged 0.004-0.005 gm. per kg. of weight. The slower method of injection employed in the present series of experiments has, therefore, increased the dosis tolerata of these mercurial compounds in an appreciable manner.

Dosis Tolerata by Intraperitoneal Injection.—With intraperitoneal injection in white mice, each dose was given according to the body weight dissolved in 1 cc of water. As shown in table 30, mercurophen was tolerated in doses as high as 0.02 gm. per kg. of body weight while with mercuric chlorid the tolerated dose was about 0.005 gm. per kg. of weight.

TABLE 30
THE TOXICITY OF MERCUROPHEN AND MERCURIC CHLORID; INTRAPERITONEAL INJECTIONS
IN MICE

Dose per Kilogram of Weight	Mercurophen						Mercuric Chlorid, Days					
	1	2	3	4	5	10	1	2	3	4	5	10
0.04	D						D					
0.035	D						D					
0.03	—	D					D					
0.025	—	—	—	—	D		D					
0.02	—	—	—	—	—	—	D					
0.015	—	—	—	—	—	—	D					
0.01	—	—	—	—	—	—	—	D				
0.008	—	—	—	—	—	—	—	—	—	D		
0.006	—	—	—	—	—	—	—	—	—	—	—	D
0.005	—	—	—	—	—	—	—	—	—	—	—	—

In our former studies we showed that the toxicity of mercurial compounds was, in general, directly proportionate to the amount of mercury contained and that on this basis of calculation the inorganic salts, as represented by the bichlorid of mercury, were no more toxic than the pseudo-complex organic combinations as represented by the succinimid and cacodylate. For this reason mercurophen with 53% mercury was not expected to be as toxic as mercuric chlorid with 74% mercury, but allowing for these differences in content of pure mercury, mercurophen is less toxic than its content in mercury would lead one

⁴ Schamberg, J. F.; Kolmer, J. A., and Raiziss, G. W.: Boston Med. and Surg. Jour., 1915, 162, p. 826.

to expect; for example, the succinimid of mercury used in this study contained 50% mercury but was distinctly more toxic than mercurio-phen containing 53% of mercury, inasmuch as the tolerated dose of the former by intravenous injection was 0.002-0.004 gm. per kg. of body weight as compared with 0.006 gm. of the latter compound. The reduction of toxicity of mercurial compounds without parallel reduction in mercury content and anti-parasiticial activity constitutes one of the chief desiderata in the experimental chemotherapy of these compounds, and the beginning appears to have been attained in sodium oxymercury orthonitro phenolate.

SPIROCHETICIDAL AND TRYPANOCIDAL ACTIVITY OF MERCUROPHEN

Spirocheticidal Activity.—The spirocheticidal activity of mercurio-phen has been tested by the intravenous and intramuscular administration of varying doses to rabbits with well developed syphilitic orchitis; in all instances 1:1,000 solutions in water were employed and the doses arranged according to the body weight of each animal.

As a general rule the intravenous injection of mercurio-phen in the dose of 0.0006 gm. per kilogram of body weight for 1 or 2 doses is followed by the disappearance of all spirochetes in the testicular lesions within 3-8 days after treatment began, followed by prompt healing of the syphiloma; with 1 or 2 intramuscular injections in amounts of 0.002 gm. per kg. of body weight the spirochetes are likewise destroyed and the lesions heal, but more slowly than is observed following intravenous medication. Our experiments have shown that mercurio-phen possesses spirochetical properties, but we have not conducted a sufficient number of comparative tests with other mercurial compounds to permit a statement being made of the relative value of mercurio-phen in the treatment of syphilitic injections. One of us (Schamberg) has injected mercurio-phen suspended in oil intramuscularly in several persons suffering with syphilis, but the local reaction was too severe to permit the continued administration of the drug by this route.

Trypanocidal Activity.—Experiments on the trypanocidal activity of mercurio-phen were conducted after methods previously described by us⁵ and consisting briefly in the injection of white rats with intraperitoneal injections of approximately known numbers *T. equiperdum* after the method of Kolmer,⁶ followed 24 hours later by the intravenous injection of graded doses of the drug dissolved in water per 100 gm. of body weight of each animal. In each experiment at least 4 control rats were included, receiving the infection alone but no drug; the strain of trypanosomes employed was of such virulence that the parasites appeared in the peripheral blood within 48 hours after infection and killed on or about the 7th day with tremendous numbers of trypanosomes in the blood. In conducting the experiments the blood of each animal was examined daily for trypanosomes and the results recorded according to the number present in a drop of blood from the tail.

⁵ Schamberg, J. F., Kolmer, J. A., and Raiziss, G. W.: Jour. Am. Med. Assn., 1915, 65, p. 2142.

⁶ Jour. Infec. Dis., 1915, 17, p. 79.

The results of one experiment with mercurophen and mercuric chlorid shown in table 31, are fairly representative of those observed in a large series of similar experiments described by us in a former communication;⁷ at best the maximum sublethal doses of mercurophen inhibited the multiplication of trypanosomes for 24 hours, but did not sterilize the animals.

TABLE 31
INFLUENCE OF MERCUROPHEN AND MERCURIC CHLORID ADMINISTERED INTRAVENOUSLY
24 HOURS AFTER INFECTION WITH T. EQUIPERDUM

Weight of Rat, Gm.	Dose per 100 Gm.	Compound	Daily Examination					
			1	2	3	4	5	6
100	0.0008	Mercurophen	—	—	D			
90	0.0007	Mercurophen	—	few	+	++	D	
95	0.0006	Mercurophen	—	few	++	++++	D	
110	0.0005	Mercurophen	—	few	++	++++	D	
120	0.0008	Mercuric chlorid	—	few	D			
115	0.0007	Mercuric chlorid	—	few	++	++++	D	
90	0.0006	Mercuric chlorid	—	few	++	++++	D	
100	0.0005	Mercuric chlorid	—	few	++	++++	++++	D
90	0	Control	—	few	++	++++	D	
120	0	Control	—	few	++	++++	++++	D

Rats infected with 110,000 trypanosomes by intraperitoneal injection.

Inasmuch as these tests were very severe by reason of employing a virulent strain of trypanosomes given a 24 hours start, further experiments were conducted in which the drugs were administered intravenously 2 hours after infection; in these tests mercurophen showed varying degrees of inhibition of multiplication of trypanosomes, in one instance the blood being kept free of parasites for 4 days.

TABLE 32
INFLUENCE OF MERCUROPHEN AND MERCURIC CHLORID ON T. EQUIPERDUM IN THE
COMBINED TEST IN VITRO-VIVO

Weight of Rats in Gm.	Dose in Gm.	Compound	Results of Daily Examination of Blood from the Tail															
			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
92	0.0005	Mercurophen	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
90	0.00025	Mercurophen	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
115	0.000125	Mercurophen	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
105	0.000062	Mercurophen	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
97	0.0005	Mercuric chlorid	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
115	0.00025	Mercuric chlorid	—	—	+	3+	3+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+
65	0.000125	Mercuric chlorid	—	—	+	4+	D											
80	0.000062	Mercuric chlorid	—	—	+	2+	2+	2+	2+	4+	D							
105	0	Control	—	—	+	4+	D											
90	0	Control	—	—	+	2+	D											

In additional experiments employing a combined in vitro-vivo technic, in which equal parts of varying dilutions of the chemical under study were mixed with blood-trypanosome emulsion and kept at 37-40 C. for fifteen minutes, when the whole or a part was injected intraperitoneally into white rats to determine the degree of trypanocidal activity, mercurophen and several other mercurial compounds were found to exercise definite trypanocidal activities as shown in the results of one experiment in table 32.

⁷ Schamberg, J. F.; Kolmer, J. A., and Raiziss, G. W.: Am. Jour. Syphilis, 1917, 1, p. 1.

The conclusions drawn by us from these experiments are to the effect that mercuriofen and other mercurial compounds as mercuric chlorid, possess some trypanocidal activity, but to a lesser degree than that exhibited by arsphenamin administered to infected rats in equal dosage per body weight.

BACTERICIDAL ACTIVITY OF MERCURIOPHEN IN VIVO

Owing to the high germicidal activity of mercuriofen in vitro in solutions containing large amounts of protein as 50% dilutions of oxalated blood and serum, we have conducted a series of experiments in rabbits to determine if a sufficient quantity of the drug could be administered intravenously to protect the animal against infection with such micro-organisms as virulent pneumococci and staphylococci, or at least impart such increased bactericidal activity to the blood as could be detected by the withdrawal of blood and the subjection of the same to bactericidal tests in vitro.*

Experiments with Pneumococci.—The virulence of a type I strain of pneumococcus for young white rats weighing from 55-90 gm. was determined by injecting various doses of 24-hour blood dextrose broth cultures intraperitoneally and intramuscularly; the minimum lethal doses of the culture by each route of infection 3 days later were as follows:

Intraperitoneal injection: 0.001 c.c.

Intramuscular injection: 0.01 c.c.

A. A series of young rats weighing from 55-70 gm. were injected intravenously with mercuriofen in dose of 0.001 gm. per kilogram of body weight 2 hours before, simultaneously with and 2 hours after infection produced by the intraperitoneal injection of 10 M. L. D's. of the culture previously determined for this route of infection. Controls received drug and culture alone.

The animals receiving culture alone died on the 3rd and 4th days; those receiving the drug alone lived indefinitely. Those receiving drug and pneumococci died on or before the 3rd day, mercuriofen in the dosage mentioned affording no protective or curative action.

B. A second series of rats of the same range in weight were injected intravenously with mercuriofen in dose of 0.001 gm. per kilogram of body weight 2 hours before, simultaneously with and 2 hours after infection produced with the intramuscular injection of 10 M. L. D's. of the culture, in order to bring about a more slowly developing infection.

The controls receiving the culture alone died 3-4 days later; those receiving drug alone lived indefinitely. Those receiving drug and pneumococci died on the 2nd and 3rd days, mercuriofen failing to afford protection and indeed, appearing to hasten death probably by the toxic influence of the drug added to the virulence of the culture.

Experiments with Staphylococci.—The virulence of a strain of Staph. aureus for white rats weighing about 90 gm. was determined by injecting increasing

* These experiments were conducted with the assistance of Dr. Edward Steinfeld.

amounts of a filtered 24-hour broth culture intravenously, necropsying all animals 3 days later and examining the viscera for abscesses. The smallest dose of the culture employed regularly producing abscesses in the kidneys, heart and diaphragm was 0.5 cc; in conducting the main experiments the culture was employed in twice this amount, or 1 cc.

The main experiments were conducted by injecting mercurophen intravenously in doses of 0.001 gm. per kg. of body weight, at the following intervals:

Two hours before infection, simultaneously with infection, 2, 4, 6 and 24 hours after infection.

As previously stated the animals were infected by injecting them intravenously with 1 cc of culture. Controls received culture alone and all developed numerous abscesses in the viscera and particularly the kidneys; controls receiving drug alone showed no changes at necropsy.

All the animals receiving mercurophen and the staphylococci after the plan given above showed abscesses in the kidneys and other organs when examined 3 days later; the number and size of these abscesses varied in the different animals but bore no relation to the administration of the drug, all experiments indicating that mercurophen in 1 dose of 0.001 gm. per kg. (equivalent to 0.06 gm. or 1 grain per 60 kg.) administered intravenously exerts no protective or curative action.

Blood Cultures.—Additional experiments were conducted with rabbits consisting in making preliminary blood cultures with 5 drops of blood from each animal in tubes of dextrose broth (10 cc) followed by the intravenous injection of 24-hour filtered broth cultures of *Staph. aureus* in varying doses, namely, 0.5, 1 and 2 cc and 10 minutes later by intravenous injection of mercurophen in dose of 0.001 gm. per kg. of body weight; a second series of animals received 0.0015 gm. mercurophen per kg. of body weight. The controls received culture alone and mercurophen alone (0.0015 gm. per kg). Blood cultures were repeated at intervals of 10 minutes, 1, 4, 24 and 48 hours; and all yielded positive cultures for staphylococci and all infected animals showed a variable number of abscesses in the kidneys on the 3rd day of the experiments.

The influence of multiple doses of mercurophen has not been tried; it is possible that in such experiments some influence may be exerted inasmuch as the following experiments show that an increased bactericidal activity of the blood may be noted soon after the administration of mercurophen, which disappears within a few hours.

Bactericidal Activity of Rabbit Serum Following the Intravenous Administration of Mercurophen.—In these experiments small amounts of blood were collected from the ear veins of rabbits and mercurophen injected intravenously followed by further bleedings at varying intervals up to 24 hours. The serums were then separated, heated at 56 C. for one-half hour and mixed in sterile test tubes in amounts of 0.2 cc with an equal quantity of a diluted 24-hour broth culture of *Staph. aureus*. After 5 hours' incubation at 37 C. the contents of each test tube were plated with 10 cc of dextrose agar at 45 C. and the plates counted after 48 hours' incubation.

The results of these experiments indicated that large doses of mercurophen imparted an increased bactericidal activity to the serum which usually had disappeared within a few hours and always at the end of 24 hours. In order to detect this increase of bactericidal activity of the blood it was necessary

to use doses of culture sufficiently small to produce a countable number of colonies when plated with agar. The results of an experiment shown in table 33 is representative of those usually observed; the greatest bactericidal activity was noted with serum collected within an hour after the administration of the mercurophen; with larger doses of culture resulting in the production of an uncountable number of colonies, this bactericidal activity was masked.

TABLE 33
BACTERICIDAL ACTIVITY OF RABBIT SERUM FOLLOWING THE INTRAVENOUS
ADMINISTRATION OF MERCUROPHEN

Dose of Mercuro- phen per Kg.	Dose of Heated Serum in C C	Dose of Culture of Staphylococci	Results of Plating			
			Before Injection	1 Hour after Injection	4 Hours after Injection	24 Hours after Injection
0.0015	0.2	0.2 c c (1:100,000)	81,000	180	205	70,000
0.0015	0.2	0.2 c c (1:10,000)	unc.	unc.	unc.	unc.

Further experiments were conducted with a multiple pipet method modified after the technic devised by Heist and Lacy and described by Heist, Cohen and Cohen⁸ for the detection of natural antibodies in the whole blood of persons and animals for pneumococci and the globoid bodies of poliomyelitis. With this technic, mercurophen, mercuric chlorid and other compounds were administered to rabbits intravenously and the bactericidal activity of the blood studied with the many stemmed pipet for staphylococci, pneumococci and *B. typhosus*. A description of the technic employed and a detailed account of the experiments are to be given elsewhere;⁹ here it may be stated that following the intravenous injection of mercurophen in dose of 0.0015 gm. per kg. of body weight, the whole uncoagulated blood was found to have an increased bactericidal activity for pneumococci, staphylococci and *B. typhosus* over a period of 1-2 hours following the injection: similar experiments with mercuric chlorid and other mercurial compounds usually failed to increase to the same degree the bactericidal activity of the uncoagulated blood of rabbits for these micro-organisms.

Influence of Mercurophen on the Total Number of Leukocytes in the Blood.—During the course of several experiments among rabbits in which mercurophen alone was administered intravenously in varying doses per kg. of body weight, total and differential leukocytic counts were made of the blood of a number of animals to determine what influence, if any, was exerted by the drug. The results of a few counts made at varying intervals after the administration of mercurophen, are shown in table 34; as a general rule, the admin-

⁸ Jour. Immunology, 1918, 3, p. 261.

⁹ Kolmer, J. A., and Trist, M. E.: A Modification of the Heist-Lacy Method for Determining the Bactericidal Action of Whole Blood in Chemotherapeutic Studies.

istration of mercurophen in dose of 0.0005-0.002 gm. per kg. of body weight in proportion of 0.001 gm in 1 cc of water, produced a slight leukocytosis with an increase of polymorphonuclear cells toward the end of the first 24 hours following the injection, with entire subsidence within 48 hours after injection.

TABLE 34
INFLUENCE OF MERCUROPHEN ADMINISTERED INTRAVENOUSLY ON THE LEUKOCYTES OF THE BLOOD

Rabbits	Weight in Gm.	Dose per Kilo in C C	Total Leukoeyte Counts				
			Before Injection	1 Hour after Injection	4 Hours after njection	24 Hours after Injection	48 Hours after Injection
1	1500	0.0005	7,800	9,600	9,200	10,600	12,800
2	1850	0.001	8,400	8,200	8,600	11,400	11,800
3	1620	0.0015	6,800	8,400	9,100	12,200	10,800
4	1700	0.002	6,200	7,200	7,600	9,800	11,600
5	1630	0.001	8,200	7,000	12,800	14,600	9,200
6	1800	water alone	8,400	8,600	9,200	8,000	8,200

THE INFLUENCE OF MERCUROPHEN ON PHAGOCYTOSIS

Since solutions of mercurophen have been used by several ophthalmologists with marked success in the treatment of pneumococcus conjunctivitis and by several surgeons in the treatment of wounds, a question of paramount importance concerns the influence of mercurophen on the phagocytic activity of polymorphonuclear leukocytes.

Experiments bearing on this problem were conducted by mixing in small sterile test tubes equal volumes of varying dilutions of mercurophen in physiologic salt solution and an emulsion of Staph. aureus followed by incubation in a water-bath for 1 hour at 37 C. when a volume of leukocytes secured from the peripheral blood of normal persons was added; after mixing, the tubes were re-incubated for an hour and stained smears prepared. Of each preparation 100 polymorphonuclear cells were counted and the percentage of phagocytes ascertained; no attempt was made to count the number of cocci within the cells. Numerous controls in which physiologic salt solution was substituted for mercurophen, were included in each experiment.

TABLE 35
THE INFLUENCE OF MERCUROPHEN ON PHAGOCYTOSIS

Experi- ment	Percentage of Leukocytes Becoming Phagocytes in Varying Final Dilutions of Mercurophen									
	1: 2,000	1: 5,000	1: 8,000	1: 10,000	1: 16,000	1: 20,000	1: 50,000	1: 80,000	1: 100,000	Con- trol
1	0.5	2	6	5	7	9	11	8	7	6
2	0.5	2	4	3	4	7	4	4	3	2

The results of two experiments are shown in table 35. With the leukocytes of one person about 6% were found to engulf the staphylococci employed; mercurophen in final dilutions up to 1:5,000 were found to inhibit phagocytosis but in higher dilutions this effect was not in evidence and some tendency toward increased phagocytosis was noted with dilutions 1:20,000 to 1:50,000. In the second experiment employing the leukocytes of a second

person and a different culture, spontaneous phagocytosis occurred with about 2% of leukocytes and mercurophen excited a similar influence.

These and additional experiments have shown, therefore, that strong solutions of mercurophen (1:1,000 to 1:5,000) may inhibit phagocytosis by a destructive influence on the leukocytes (fragmentation of protoplasm and alteration in staining), whereas higher dilutions do not exert this destructive influence and appear to enhance phagocytosis to a slight degree.

TABLE 36
SUMMARY SHOWING RESULTS OF COMPARATIVE TESTS WITH MERCUROPHEN AND MERCURIC CHLORID

Tests	Comparative Results and Properties	
	Mercurophen	Mercuric Chlorid
Highest antiseptic dilution for B. typhosus	1:480,000	1:540,000
Highest antiseptic dilution for Staph. aureus	1:1,200,000	1:540,000
Highest antiseptic dilution for B. anthracis	1:1,400,000	1:280,000
Phenol coefficient in Hygienic Laboratory test.....	900 to 1300	840
Rapidity of germicidal activity for staphylococci	1:100,000 (1 hour)	1:100,000 (more than 2 hr.)
Rapidity of germicidal activity for typhoid bacilli.....	1:100,000 (15 minutes)	1:100,000 (60 minutes)
Stability of solutions.....	Stable	Stable
Germicidal activity in blood.....	1:2,000 (60 minutes)	1:2,000 (more than 2 hr.)
Germicidal activity in ascites fluid.....	1:4,000 (1 minute)	1:4,000 (more than 10 min.)
Germicidal activity in serum.....	1:10,000 (1 minute)	1:10,000 (more than 30 min.)
Precipitation of serum proteins.....	Not in 1:200	Positive in 1:1,000
Disinfection of typhoid urine.....	1:8,000 (5 minutes)	1:8,000 (30 minutes)
Disinfection of ordinary urine.....	1:2,000 (5 minutes)	1:2,000 (60 minutes)
Disinfection of feces	1:1,000 (10 minutes)	1:1,000 (more than 10 min.)
Disinfection of sputum	1:4,000 (few minutes)	1:4,000 (more than 30 min.)
Disinfection of rubber gloves.....	1:5,000 (1 minute)	1:5,000 (1 to 3 minutes)
Disinfection of catheters	1:5,000 (8 minutes)	1:8,000 (10 minutes)
Disinfection of instruments	1:10,000 (1 to 5 min.)	1:10,000 (more than 15 min.)
Tarnishing of instruments.....	Absent	Marked
Disinfection of skin.....	1:5,000 (1 to 3 min.)	1:5,000 (5 to 7 min.)
Disinfection of skin.....	1:1,000 (1 minute)	1:1,000 (5 minutes)
Irritant action of skin.....	Absent	May occur
Irritant action on eye.....	Very slight	Marked
Disinfection of pus.....	1:2,000 (5 minutes)	1:2,000 (15 minutes)
Germicidal activity of impregnated gauze.....	1:5,000 (1 minute)	1:5,000 (30 minutes)
Dosis tolerata by intravenous administration.....	0.008 gm. per kilo	0.003 to 0.004 gm. per kilo
Dosis tolerata by intraperitoneal administration.....	0.02 gm. per kilo	0.005 gm. per kilo
Spirocheticidal activity	Present	Present
Trypanocidal activity	Present in greater deg.	Present
Protection against staphylococcus infection.....	None	None
Protection against pneumococcus infection.....	None	None
Bactericidal action of blood after administration.....	Present in greater deg.	Practically absent
Influence of phagocytosis.....	Inhibitory up to 1:5,000	0
Influence of leukocyte counts.....	Slightly stimulating	0

SUMMARY

Throughout this study mercuric chlorid was employed for comparative tests with mercurophen by reason of the fact that the properties of the former are so well known and its being the most widely used of the available mercurial preparations. Since the tech-

nic of a disinfectant test exerts great influence on the results, we have attempted to simplify our experiments as much as possible, and in every instance the experiments with mercurophen were conducted simultaneously with mercuric chlorid and other mercurial compounds in order that the same technic might be applied to both and the results be strictly comparative.

The technic employed in the various tests and the results observed with mercurophen, mercuric chlorid, other mercurial compounds and phenol are given in the body of this communication; a summary is given in table 36 which shows at a glance the results of comparative tests with mercurophen containing 53% mercury and mercuric chlorid containing about 74% mercury.

CONCLUSIONS

Sodium oxymercury-orthonitrate-phenolate, for convenience designated by us as mercurophen, possess certain superior properties over other mercurial compounds, as follows:

In germicidal activity mercurophen is equal or superior to mercuric chlorid and other mercurial compounds containing more mercury; inasmuch as the germicidal activity of this class of compounds bears a relation to their content in mercury and the electrolytic dissociation of the mercury compound in watery solution, mercurophen is of special interest from the chemotherapeutic standpoint.

Mercurophen is generally more rapid in its germicidal activity than other mercurial compounds.

Mercurophen appears to possess a special destructive affinity for cocci and spore-forming bacilli.

Mercurophen has proven superior to mercuric chlorid in the disinfection of urine, feces, sputum, pus, catheters, instruments, rubber gloves and the skin.

Mercurophen maintains a higher degree of germicidal activity in blood serum than mercurial chlorid.

Mercurophen does not precipitate protein in as high concentration as 1:100; nor does it irritate the skin or tarnish surgical instruments.

Mercurophen is somewhat more trypanocidal than mercuric chlorid and is capable of temporarily raising the bactericidal action of the blood after intravenous administration.

In terms of mercury, mercurophen is less toxic for animals than other soluble mercurial compounds and this constitutes a fact of much importance in the chemotherapy of this class of compounds.

SERUM STUDIES ON THE ETIOLOGY OF INFLUENZA

JOHN A. KOLMER, MARY E. TRIST, AND ELIZABETH YAGLE

From the Pathological Laboratories of the Graduate School of Medicine of the University of Pennsylvania and the Philadelphia Hospital for Contagious Diseases

Until the recent pandemic, the bacillus of influenza was quite generally accepted as the cause of this disease, although the evidence never has been conclusive. The investigations of Wollstein¹ and Davis² have shown that bacilli resembling the influenza bacillus are found in the upper respiratory tract of persons suffering not only from influenza but from other diseases, so that these bacilli may be considered as widely distributed. At the present time much confusion exists in the results of bacteriologic examinations of the secretions and lesions of the respiratory tract of persons suffering and succumbing to influenza and owing to the difficulties encountered in the isolation of *B. influenzae* until considerable experience has been gained, such discrepancies in results are to be expected. Many regard the disease as due to *B. influenza* with streptococci, pneumococci, *M. catarrhalis* and staphylococci as organisms of secondary infection, one or more assuming importance according to their geographical distribution. However, opinion is steadily gaining to the effect that the cause of influenza has not been discovered and that the influenza bacillus is to be classed with the secondary organisms but probably occupying chief place among them and capable of producing bronchitis and bronchopneumonia either alone or in conjunction with streptococci, pneumococci, *M. catarrhalis*, staphylococci and *B. mucosus*.

Opinions regarding the relationship of these various bacteria to influenza have been based mainly on bacteriologic examinations of the sputum of persons suffering with influenza and to a lesser extent on examination of the bronchi and lungs of fatal cases; owing to the wide distribution of streptococci, pneumococci (particularly type 4), *M. catarrhalis*, staphylococci and bacilli similar to the influenza bacillus in the mouth and upper air passages of most persons irrespective of

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¹ Jour. Exper. Med., 1906, 8, p. 681.

² Jour. Infect. Dis., 1907, 4, p. 73.

influenza, the exact rôle they exert in the infection is difficult to evaluate. For this reason we have made certain serum studies in influenza for the purpose of determining whether or not antibodies were produced by these various organisms (excepting pneumococci) with the hope that the results under limitations mentioned later, would throw additional light on the etiology of the disease.

Little is known of the immunity response to the influenza bacillus except that agglutinins appear in the blood rather tardily; Wollstein¹ found agglutinin in the serum of two cases of influenza after the 8th day of the disease, but further investigations do not appear to have been made.

Our main purpose was to determine whether antibodies for *B. influenzae* occur normally in the serum and the degree and kind of antibody production for this bacillus during influenza. Similar studies were made with streptococci, *M. catarrhalis*, staphylococci and other organisms isolated from persons suffering from or succumbing to influenza, on the basis that such immunological examinations may show in a broad and general manner which of these organisms had assumed sufficient pathogenicity in Philadelphia to stimulate antibody production.

The serums of 9 healthy adults varying in age from 20-32 years, who had escaped influenza and had not received any vaccine, and the serums of 31 adults in varying stages of influenza were tested for the presence of thermostabile opsonin, agglutinin and complement fixing antibody with various bacterial antigens; comparison is made of the results obtained with serums of healthy persons and with those obtained in varying stages of influenza inasmuch as opportunity was not afforded for the examination of the serums of persons prior to their illness or at intervals during the attack of the disease.

ANTIGENS

All of the antigens were polyvalent, prepared of a varying number of cultures of the respective organisms freshly isolated from the secretions and lesions of the upper respiratory tract of persons from the 4th to the 9th day of the disease, as follows:

1. Streptococcus antigen was prepared of cultures of freshly isolated hemolytic streptococci from 8 persons.
2. Catarrhalis antigen was prepared of cultures of freshly isolated micrococci from 7 persons.

3. Staphylococcus antigen was prepared of cultures of Staph. aureus from 16 persons.

4. Pseudodiphtheria antigen was prepared of cultures from 6 persons.

5. Influenza antigen was prepared of cultures of gram-negative, nonmotile, nonspore forming, pleomorphic and *absolute* hemophilic bacilli from 4 different persons.

6. Wassermann reactions were made with each serum, using for antigen an alcoholic extract of beef heart reenforced with cholesterin.

RESULTS OF COMPLEMENT FIXATION

All of the antigens were prepared by suspending the various bacteria in normal salt solution followed by heating at 60 C. for 1 hour and titration for anticomplementary activity. In the fixation tests each antigen was employed in a dose equivalent to one-third the anticomplementary unit.

The antigenic activity of all antigens was highest when freshly prepared; antigens preserved in a refrigerator for 4 weeks had lost antigenic sensitiveness to a marked degree.

All serums were heated at 56 C. for one-half hour and used in a dose of 0.2 c c.

An antishoop hemolytic system was employed and all tests were conducted in duplicate, one set being incubated for 1 hour in a water-bath at 38 C. and the second in a refrigerator at 5-12 C. for at least 12 hours prior to the addition of hemolysin and indicator antigen.

In all experiments the usual controls were included and the results read and recorded immediately after the second period of incubation and complete hemolysis of the controls.

RESULTS WITH SERUMS OF HEALTHY PERSONS

The results with the serum of 9 Wassermann negative and healthy adults who had never had influenza insofar as they knew and 2 of whom contracted the disease subsequent to these tests were all wholly negative and indicate the absence of complement fixing for the antigens employed; particular interest was paid the antigen of B. influenzae.

RESULTS WITH SERUMS OF PERSONS SUFFERING WITH INFLUENZA

The results of complement fixation tests with the serums of 31 persons in the various stages of acute influenza are shown in tables 1 and 2.

As far as could be ascertained, all of these persons were suffering with a primary attack of the disease.

The word "none" under complications as listed in the tables is used in a purely clinical sense and indicates that at the time blood was drawn for these tests the attending physicians could not detect signs of pneumonia or other complications; the term "pneumonia" refers to the bronchopneumonia of special characteristics so prevalent during this pandemic.

The earliest test was on the 3rd day after the onset of symptoms and the last 5 weeks after the onset.

The results may be summarized as follows:

With the polyvalent antigen of *B. influenzae* positive reactions resulted with 16 serums (51%) in the water-bath series and with 14 serums (45%) in the refrigerator series. The earliest positive reaction observed occurred on the 4th day of the disease.

With the polyvalent antigens of hemolytic streptococci and *Micrococcus catarrhalis* positive reactions resulted with about 38% of serums with each antigen.

With the polyvalent antigens of staphylococcus and pseudodiphtheria bacillus the reactions were generally negative. One patient on the 15th day of the disease yielded a weakly positive reaction with the staphylococcus antigen.

RESULTS OF PHAGOCYTOSIS TESTS

In these experiments the various bacterial emulsions were polyvalent and prepared by suspending the respective organisms in normal salt solution in proper density and free of clumps. Freshly prepared washed human leukocytes from healthy laboratory assistants were employed; all serums were heated at 56 C. for half an hour prior to the tests which were made in capillary pipets after the method of Wright and incubated for half an hour when duplicate smears were prepared.

In order to reduce the degree of error consequent to variation in the leukocytes and cultures, all serums were carefully preserved after

TABLE 1
RESULTS OF COMPLEMENT FIXATION TESTS IN THE FIRST WEEK OF INFLUENZA

[illegible]

* Had two doses of mixed vaccine within a few days before onset of influenza.

TABLE 2
RESULTS OF COMPLEMENT FIXATION TESTS IN INFLUENZA AFTER THE FIRST WEEK OF THE DISEASE

[illegible]

collection and the phagocytosis tests conducted with heated serums for thermostable opsonins (at the same time) so that the results would be strictly comparable.

In the smears from 50-100 polymorphonuclear leukocytes were examined and the percentage of phagocytes and average number of bacteria per phagocyte recorded. The degree of spontaneous phagocytosis occurring with each antigen is shown in table 3.

RESULTS WITH THE SERUMS OF HEALTHY PERSONS

The results observed with the serums of 9 healthy adults are shown in table 3 and summarized in table 6.

TABLE 3
RESULTS OF PHAGOCYTOSIS TESTS WITH SERUMS OF HEALTHY PERSONS

Serum	Streptococci		M. catarrhalis		Staphylococci		Pseudodiphtheria Bacillus		B. influenzae	
	Percentage of Phagocytes	Average No. of Bacteria per Phagocyte	Percentage of Phagocytes	Average No. of Bacteria per Phagocyte	Percentage of Phagocytes	Average No. of Bacteria per Phagocyte	Percentage of Phagocytes	Average No. of Bacteria per Phagocyte	Percentage of Phagocytes	Average No. of Bacteria per Phagocyte
1	4	0.4	6	4.0	16	4.0	2	2.0	6	2.0
2	4	0.8	10	3.0	36	4.2	12	3.8	6	1.5
3	6	2.0	20	3.0	42	4.0	22	3.0	6	1.2
4	12	2.0	26	2.5	32	3.0	16	2.0	8	2.0
5	24	2.2	26	1.4	40	3.0	16	1.5	16	1.3
6	12	2.2	26	2.0	44	3.4	12	2.4	10	2.0
7	18	2.0	18	1.8	34	2.4	10	1.6	12	1.1
8	16	1.6	26	2.0	22	3.0	18	1.6	10	1.4
9	12	1.5	20	2.4	42	4.0	10	1.2	6	2.4
Control	8	1.4	6	1.8	12	1.5	4	0.8	8	2.0
Control	6	1.2	10	2.0	16	1.4	4	0.6	10	1.8

As shown in table 4 considerable variation in the percentage of leukocytes becoming phagocytes were found to occur; similar variations were found in the number of bacteria engulfed. For this reason an accurate analysis cannot be made, but the results interpreted by viewing the whole, in which the taking of averages aids slightly in evaluation. Much better data could have been secured had it been possible for us to secure and study the serums of persons before and at intervals during the illness with influenza.³

³ Turnidiff (Phagocytic Experiments in Influenza, Jour. Am. Med. Assn., 1918, 71, p. 1733) reports that specific opsonins for a green-producing streptococcus isolated by Mathers, developed during the course of the influenza. A specific decrease in opsonins for this organism occurred in pneumonia following influenza, which persisted until recovery when a rise above normal occurred. No fluctuations in opsonin were observed with Bacillus influenzae, Micrococcus catarrhalis or hemolytic streptococcus.

With the polyvalent antigen of *B. influenzae* from 4-16% of leukocytes were found to engulf the bacilli, which degree of phagocytosis was quite similar to that observed spontaneously in the absence of serum. According to our results normal human serum contains none or but traces of thermostabile opsonin for the influenza bacillus.

About 60% of serums of healthy persons were found to contain slight amounts of thermostabile opsonin for the hemolytic streptococci employed in these tests, and the majority contained traces also for *M. catarrhalis*, *Staph. aureus* and pseudodiphtheria bacilli.

RESULTS WITH THE SERUMS OF PERSONS SUFFERING WITH INFLUENZA

The results of phagocytosis tests with the serums of 19 persons varying from the 3rd to the 25th day of the disease, are shown in tables 4 and 5; of these 19 patients, 9 were regarded clinically as having bronchopneumonia at the time the specimens of blood were taken for these tests.

As previously stated considerable variation in results was observed, but it would appear that a slight increase of thermostabile opsonin for *B. influenzae* occurs in the majority of persons and particularly after the first week of influenza; with the serums of persons suffering with bronchopneumonia, less phagocytosis was generally observed.

Thermostabile opsonins for hemolytic streptococci were generally increased and particularly during the first week of influenza and before the onset of bronchopneumonia; with the serums of persons suffering with pneumonia a general decrease in opsonin content was apparent.

The amounts of thermostabile opsonins for *M. catarrhalis*, *Staph. aureus* and pseudodiphtheria bacilli in the serums of influenza patients were practically the same as found in the serums of healthy adults.

RESULTS OF AGGLUTINATION TESTS

Microscopic agglutination tests were made with a number of heated serums from healthy persons; and tests with influenza serums were made with the same polyvalent bacterial antigens employed in the complement fixation tests.

In making these tests the mixtures of serum and bacterial emulsions were incubated at 55 C. on a water-bath for 2 hours and read

TABLE 4
RESULTS OF PHAGOCYTOSIS TESTS WITH SERUMS OF PERSONS IN FIRST WEEK OF INFLUENZA

Day of Disease	Complica- tions	Streptococci		M. catarrhalis		Staphylococci		Pseudodiphtheria Bac.		B. influenzae	
		Percentage Phagocytes	Average Number of Bacteria per Phagocyte	Percentage Phagocytes	Average Number of Bacteria per Phagocyte	Percentage Phagocytes	Average Number of Bacteria per Phagocyte	Percentage Phagocytes	Average Number of Bacteria per Phagocyte	Percentage Phagocytes	Average Number of Bacteria per Phagocyte
5	None	34	2.4	22	1.5	28	3.5	10	3.0	22	2.0
5	None	20	1.3	6	4.0	32	3.3	14	3.0	10	1.6
3	None	10	2.6	24	3.0	40	4.0	6	1.3	12	1.3
6	None	18	2.2	20	3.3	30	2.6	18	3.0	12	2.6
7	Pneumonia	15	2.0	4	2.0	20	4.0	4	1.3	8	2.4
7	None	16	1.5	32	0.6	16	1.5	20	0.8	12	1.5
7	None	22	1.0	16	0.9	16	1.0	22	0.9	18	1.0

TABLE 5
RESULTS OF PHAGOCYTOSIS TESTS WITH SERUMS OF PERSONS IN THE SECOND TO FOURTH WEEKS OF INFLUENZA

Day of Disease	Complica- tions	Streptococci		M. catarrhalis		Staphylococci		Pseudodiphtheria Bac.		B. influenzae	
		Percentage Phagocytes	Average Number of Bacteria per Phagocyte	Percentage Phagocytes	Average Number of Bacteria per Phagocyte	Percentage Phagocytes	Average Number of Bacteria per Phagocyte	Percentage Phagocytes	Average Number of Bacteria per Phagocyte	Percentage Phagocytes	Average Number of Bacteria per Phagocyte
10	None	28	1.5	30	2.1	44	3.0	20	1.0	38	1.5
11	None	34	5.0	20	5.9	44	3.0	20	3.0	18	4.0
10	Pneumonia	8	3.0	20	3.0	30	5.0	14	4.0	14	1.5
16	Pneumonia	8	2.5	6	1.8	8	3.0	6	2.0	6	3.0
25	Pneumonia	8	2.0	8	1.5	14	2.5	8	2.0	14	2.0
20	Pneumonia	6	1.0	10	2.6	22	4.0	10	1.0	12	3.0
17	Pneumonia	8	4.0	16	4.0	30	3.5	8	1.5	4	1.0
21	Pneumonia	16	3.0	18	1.6	26	5.0	8	4.0	4	2.0
18	Pneumonia	6	1.8	12	1.5	26	4.0	4	2.0	14	2.0
21	Pneumonia	18	1.4	10	1.0	20	2.4	16	2.0	10	2.0
15	None	12	1.4	12	0.8	24	1.6	14	1.0	16	1.0
9	None	28	1.2	34	1.5	14	2.0	14	1.0	20	1.3

TABLE 6
SUMMARY OF PHAGOCYTOSIS TESTS SHOWING THE PERCENTAGE OF PHAGOCYTES WITH EACH ANTIGEN AND VARIOUS SERUMS

Serums	Total Tested	Streptococci			M. catarrhalis			Staphylococci			Pseudodiphtheria Bac.			B. influenzae		
		Highest	Lowest	Aver.	Highest	Lowest	Aver.	Highest	Lowest	Aver.	Highest	Lowest	Aver.	Highest	Lowest	Aver.
Healthy persons.....	9	24	4	12	26	6	20	44	16	34	18	2	13	16	6	8
1st week of influenza....	7	34	10	19	32	4	18	40	16	26	20	4	13	22	8	13
2nd-4th wk. of influenza	12	34	6	15	34	6	16	44	8	25	20	4	12	38	4	4
No serum.....	2	8	6	7	10	6	8	16	12	14	4	4	4	10	8	9

after standing in a refrigerator over night. The usual salt solution controls were included.

Microscopic tests were made also but the majority of cultures of *B. influenzae*, streptococci and *M. catarrhalis* showed sufficient spontaneous agglutination to interfere with the interpretation of these tests.

The results observed may be summarized as follows:

The heated serum of the majority of normal healthy adults did not agglutinate the polyvalent antigen of *B. influenzae* even in dilutions as low as 1:2; a few serums produced partial agglutination in dilution of 1:2 but not in higher dilutions (table 7).

Similar results with heated normal serums were observed with the polyvalent antigens of hemolytic streptococci, *M. catarrhalis*, *Staph. aureus* and pseudodiphtheria bacillus.

TABLE 7
RESULTS OF MACROSCOPIC AGGLUTINATION TESTS WITH POLYVALENT ANTIGEN OF
B. INFLUENZAE

Serum	Final Dilutions					
	1:2	1:4	1:8	1:16	1:32	1:64
Healthy adult control.....	—	—	—	—	—	—
Healthy adult control.....	±	—	—	—	—	—
Healthy adult control.....	±	—	—	—	—	—
Healthy adult control.....	±	—	—	—	—	—
Influenza; 4th day.....	—	—	—	—	—	—
Influenza; 5th day.....	±	—	—	—	—	—
Influenza; 5th day.....	—	—	—	—	—	—
Influenza; 11th day.....	+	+	—	—	—	—
Influenza; 16th day.....	+	+	+	—	—	—
Influenza; 16th day.....	+	+	+	+	+	—
Influenza; 25th day.....	+	+	+	—	—	—

Appreciable amounts of agglutinin for *B. influenzae* was found in about 30% of serums from persons suffering with influenza after the 1st week of the disease; as a general rule the limits of agglutination were found in dilutions 1:8 to 1:32 but with the serums of 2 persons who had suffered with a bronchopneumonia agglutination occurred in dilutions as high as 1:64.

The serums of 2 persons suffering with bronchopneumonia tested on the 15th and 18th days of illness, respectively, agglutinated the polyvalent antigen of hemolytic streptococci in dilutions up to 1:4, but tests with the remaining serums yielded negative results.

All tests with polyvalent antigens of *M. catarrhalis*, *Staph. aureus* and pseudodiphtheria bacillus yielded negative results.

DISCUSSION

The general results indicate that antibodies and particularly the complement fixing body, are developed in the serums of most influenza patients for *B. influenzae* and to a lesser extent for hemolytic streptococci and *M. catarrhalis*; similar studies were not made with pneumococci. These results, however, cannot be interpreted as an indication that the bacillus of influenza is the primary or chief cause of influenza inasmuch as this bacillus very probably possesses in the majority of cases sufficient pathogenicity to stimulate antibody production as an organism of secondary infection; similar studies with the streptococci of scarlet fever and the micrococci of acute anterior poliomyelitis indicate that what are very probably organisms of secondary infection, may stimulate specific antibody production.

In general, the results of this investigation indicate on the basis of antibody production by the various organisms studied, that the bacillus of influenza produces most antibody during the course of the disease, and if this bacillus is not the actual or primary cause of the infection it is at least the chief organism of secondary infection, with streptococci ranking second in antibody production and importance.

SUMMARY

Complement fixation, phagocytosis and agglutination tests were made with the serums of healthy adults who had never had influenza and with the serums of persons in varying stages of influenza and bronchopneumonia, with polyvalent antigens of *B. influenzae*, hemolytic streptococci, *M. catarrhalis*, *Staph. aureus* and pseudodiphtheria bacillus.

The serums of normal healthy adults did not fix complement with the polyvalent antigen of *B. influenzae* and the majority were free of thermostabile opsonin and agglutinin for these bacilli.

The serums of from 45-50% of persons suffering with influenza yielded complement fixation with the polyvalent antigen of *B. influenzae*.

A slight increase of thermostabile opsonin for *B. influenzae* was found with the majority of serums and particularly after the 1st week of the disease; during bronchopneumonia a decrease in opsonic activity of the serum was generally apparent.

Slight amounts of agglutinin for a polyvalent antigen of *B. influenzae* was found in about 30% of serums of persons suffering with influenza after the 1st week of the disease.

The serums of normal healthy adults yielded no complement fixation with polyvalent antigen of hemolytic streptococci; positive reactions were observed with about 38% of serums from persons with influenza.

A slight increase of thermostabile opsonin for hemolytic streptococci generally occurred during the 1st week of influenza with a decrease during the period of pneumonia; an increase of agglutinin was not apparent.

Complement fixation was not obtained with the serums of normal healthy adults and a polyvalent antigen of *M. catarrhalis*; about 38% of the serums of persons suffering with influenza yielded positive reactions. As a general rule, no increase of thermostabile opsonin or agglutinin for this antigen was detected in the serums of persons with influenza.

Complement fixation tests with the serums of normal healthy adults yielded negative reactions with the polyvalent antigens of *Staph. aureus* and *pseudodiphtheria bacillus*; the serums of persons suffering with influenza likewise generally yielded negative reactions and did not show appreciable increase in thermostabile opsonin and agglutinin for these antigens.

The general results of this investigation have shown that in Philadelphia, as a result of influenza, most antibody production occurred for the *B. influenzae* indicating that this bacillus very probably exerted the most important rôle in the disease as an organism of secondary infection if not the actual or primary etiologic agent; streptococci and *M. catarrhalis* were also sufficiently pathogenic to produce appreciable amounts of antibodies in the serums of many persons suffering or convalescent from influenza.

AN EPIDEMIC OF PNEUMOCOCCUS BRONCHO-PNEUMONIA *

EDWIN F. HIRSCH AND MARION MCKINNEY

From the Laboratory of the Base Hospital, Camp Grant, Ill.

INTRODUCTION

An epidemic disease characterized by a sore or dry throat, cough, fever, general prostration, and in a certain number of patients by a rapidly developing pneumonia broke out at Camp Grant, Ill., Sept. 21, 1918. The hospital admissions increased rapidly, attained their maximum between Sept. 26 and Oct. 2, and then gradually diminished until by Oct. 18, they had reached about the usual daily hospital admission rate. During the period between Sept. 21 and Oct. 18, 9,037 patients were admitted to the hospital. While all of the admissions were not the direct result of the epidemic, the proportion of those not so entering was extremely small. During the several days immediately preceding the onset of the epidemic a few patients, not more than 15 or 20, were admitted with the diagnosis of influenza, and after Oct. 18 others were admitted with a similar diagnosis, but not in as large numbers nor as seriously ill, and during the epidemic proper some of the mildly ill patients were treated and quartered in the infirmaries. The number of hospital admissions mentioned, therefore, does not account accurately for all those sick with the epidemic disease.

The disease began suddenly, commonly within a period of 10-12 hours from a state of comparative well being. Some patients said that they had coughed or had had a cold for several days preceding the acute onset of illness. Prostration was a striking manifestation, many coughed, all had fever, and those with moderately severe nasopharyngeal or bronchial manifestations had a peculiar dusky, almost cyanotic, flushed face, particularly the forehead, cheeks, and lips.

Pneumonia rapidly became the serious consequence in the epidemic, and its high mortality one of the most baffling factors so far as the medical management of patients was concerned. Many patients were in the hospital but a short time before death, and during this interval had extreme respiratory difficulty, with marked cyanosis of the face and body generally. Some became moderately or intensely jaundiced early in the disease, a circumstance that

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suggested strongly a blood invasion by some pathogenic organism. The total number of pneumonia patients registered in the hospital from Sept. 20 to Oct. 17 is 2,371.

The first death occurred on the 3rd day after the explosive onset of the epidemic in a patient with a diagnosis of pneumonia. From Sept. 21 to Oct. 18 there were 1,019 deaths, and from the latter date to Oct. 31 there were 51. Almost every death resulted from pneumonia, although during the latter part of the epidemic, complications and sequelae of an active or healing pneumonia contributed no small part in continuing the mortality. In addition to these, infections of the leptomeninges, sinuses, and other bony spaces of the head became of decided importance.

The arrival of a large number of patients in the hospital with symptoms referable to infection of the respiratory passages suggested at once that the possible or even highly probable portal of entry of the disease-producing organism was the nasopharynx, and that a bacteriologic examination of the nasopharynx would demonstrate the variety of bacteria as well as the predominating organism, if there were any such. This contention was further supported by the fact that the pharyngeal tissues were edematous, reddened, and otherwise obviously involved in the infection.

From about the first 300 patients admitted, throat cultures were made on Loeffler's medium, and having determined after 24 hours' incubation that the predominating organisms growing on this medium were gram-positive, usually diplococci as such or in short chains, about 80 throat cultures were made as surface streaks on dextrose blood-agar plates. This medium, it was thought, would aid materially in differentiating the gram-positive organisms, as well as furnish a favorable substrate for the growth of such gram-negative bacilli as *B. influenzae*. In order to make this contention more secure, if possible, another series of surface inoculations was made on plain blood-agar plates from about 90 more patients. All of these cultures were made on the second and third days of the epidemic — practically at its onset.

Appreciating further that a pneumonic process in all probability would follow in a certain number of patients, and that careful bacteriologic examinations would be vitally important in the postmortem studies, a systematic plan of investigation was laid out and followed rigidly. This plan is detailed as followed:

1. To culture in dextrose broth, blood removed from the heart with a sterile pipet.
2. To inoculate in surface streaks on plain blood-agar plates the exudates from consolidated lung tissue and from bronchi.

3. To make direct smears on glass slides of such exudates for comparison with (2), stained according to the Gram's method (counterstained with dilute aqueous fuchsin).

4. To culture pleural, pericardial, spinal, and other fluids or exudates in dextrose broth or on plain blood-agar plates.

5. To make surface streaks on plain blood-agar plates of exudates found in the sinuses of the head or middle ears.

6. To isolate in pure culture and identify the organisms found in the diseased tissues, and in the various body fluids and exudates in so far as is possible.

TABLE 1
PNEUMOCOCCI FROM THROAT CULTURES

Culture	Inulin Fermentation	Capsule		Bile Solubility	Amount of 24-Hour Blood-Agar Slant Injected into White Mouse	Dose Fatal in Hours	Type	Organism Recovered Pure from Mouse Heart Blood
		On Blood Agar	In Heart Blood of Mouse					
4	—	—	+	+	0.166	24	IV	+
6	—	+	+	+	0.166	34	II	+
8	—	—	+	+	0.166	20	II	+
11	—	—	+	+	0.166	18	IV	+
12	—	—	—	+	0.166	21	IV	+
15	—	—	—	+	0.166	20	IV	+
16	—	—	+	+	0.166	20	II	+
18	+	—	—	+	0.166	18	IV	+

+ positive; — negative.

7. To fix in Zenker's solution tissues for microscopic study, as well as to preserve gross specimens for museum demonstration.

8. Having determined the predominating organism, to test its pathogenicity in susceptible, moderately susceptible, and resistant laboratory animals.

This much of a plan was laid down early, and carefully followed. Later additional methods of investigation were developed, among which are blood cultures of patients in the hospital. In the cultures of all exudates, recently prepared, plain blood-agar plates were used, and usually controlled by direct smears on glass slides stained by Gram's method.

The isolation and identification of bacteria were carried out according to recognized standard methods, the details of which appear later.

THROAT CULTURES

The frequency of nasopharyngeal inflammation in the early admissions to the hospital prompted the making of cultures on Loeffler's medium from the first 297 patients received. These cultures were taken in the usual way with

sterile cotton applicators, the secretions collected on the cotton inoculated at once on the surface of the medium. After about 24 hours' incubation, smears of the surface growth were made on glass slides, fixed, and stained according to Gram's method, counterstaining with a dilute aqueous solution of fuchsin. The results of the microscopic examination of these cultures are given in the following table according to the predominating or mixed flora noted:

Gram-positive diplococcus.....	189
Gram-positive diplococcus and staphylococcus.....	6
Gram-positive diplococcus and gram-negative bacillus.....	1
Gram-positive diplococcus and gram-negative coccus.....	4
Gram-positive diplococcus and gram-positive bacillus.....	3
Staphylococcus	30
Gram-negative bacillus.....	1
Gram-negative coccus.....	6
Gram-negative bacillus and gram-positive bacillus.....	1
Gram-positive bacillus.....	8
No growth.....	48

This early survey demonstrated a remarkably high frequency of gram-positive, frequently lancet-shaped diplococci as such or in short chains in cultures from the posterior pharynx of these patients. It also disclosed a relative infrequency of gram-negative organisms of all kinds, presuming of course that the substrate favored their growth. Not feeling entirely certain of this, and wishing to differentiate still further the variety of gram-positive organisms on culture mediums, a series of 71 throat cultures was taken on dextrose blood-agar plates. This was done on the second and third days of the epidemic. After 24 hours' incubation practically every plate contained fine, moist, transparent or nearly so, slightly umbilicated colonies of about $\frac{1}{2}$ mm. diameter surrounded by or contained in a distinct zone of green. Many of these plates were pure cultures of such colonies containing gram-positive diplococci, commonly lancet-shaped, and sometimes arranged in short chains. Another series of 88 plain blood-agar plates was prepared from other patients in the hospital, and these also contained many fine green colonies, frequently such almost exclusively. All of the blood-agar plates were searched carefully for gram negative bacilli, but these were found only occasionally, and never as a predominating organism in any culture.

With the purpose of determining more closely the presence of gram-negative bacilli in throat cultures, sterile cotton applicators used to collect the secretions from the posterior pharynx were shaken out in broth tubes, and from the broth dilution plain blood-agar plates were poured. Fourteen such plates were made, each one controlled by surface inoculation on plain blood agar plates of the undiluted secretions, and colonies of gram-negative bacilli were found in no large numbers in three, and always in plates where the predominating organism was a gram-positive diplococcus.

Colonies of hemolytic streptococci appeared occasionally in 20 of the 159 blood-agar plates inoculated with secretions from the posterior pharynx, non-hemolytic in not more than 13. During the examination of the throat cultures on blood-agar plates, many strains of gram-positive diplococci were isolated, and table 1 lists a few, giving their morphology, cultural characteristics, type, and approximate virulence.

ANATOMIC CHANGES

Early involvement of the lungs seemed an inevitable consequence with an upper respiratory tract infection accompanied by such severe prostration, and as a matter of fact, patients came to the hospital with a well developed pneumonia. On the third day of the epidemic proper the first death occurred with the diagnosis of pneumonia and with few exceptions this was the clinical diagnosis of other patients dying in the hospital during the epidemic. Those others not so diagnosed died from sequelae or complications of a lung infection, or from complications of a cranial sinus infection or middle ear disease.

TABLE 2
PNEUMOCOCCI FROM HEART BLOOD POSTMORTUM

Culture	Inulin Fermentation	Capsule		Bile Solubility	Amount of 24-Hour Blood-Agar Slant Injected into White Mouse	Dose Fatal in Hours	Type	Organism Recovered Pure from Mouse Heart Blood
		On Blood Agar	In Heart Blood of Mouse					
1001	+	—	+	+	0.0231	11	II	+
1002	+	—	+	+	0.0231	11	II	+
1005	+	—	+	+	0.0231	17	IV	+
1011	+	—	+	+	0.0231	23	II	+
1013	+	—	+	+	0.0231	18	IV	+
1016	+	—	+	+	0.0231	13	IV	+
1017	+	—	+	+	0.0231	11	II	+
1019	+	—	+	+	0.0231	13	II	+
1020	+	+	+	+	0.0231	11	II	+
1021	+	—	+	+	0.0231	11	IV	+
1037	—	—	+	+	0.0231	27	II	+
1038	+	+	+	+	0.0231	25	II	+
1040	+	—	+	+	0.0231	25	IV	+
1048	+	—	+	+	0.0231	36	IV	+
1049	+	—	+	+	0.0231	16	II	+
1057	+	—	+	+	0.0231	32	I	+
1061	+	—	+	+	0.0231	32	II	+
1066	+	—	+	+	0.0231	24	I	+
1069	+	—	+	+	0.0231	18	II	+
1072	+	—	+	+	0.0231	24	II	+

+ positive; — negative.

During the epidemic 198 postmortem examinations were made. Those held at the beginning included an examination of all the organs of the chest and abdomen as well as the head, but later when the death rate was highest only the organs of the chest were examined. The changes in those dying late in the epidemic deviated only in degree and progress rather than in kind from those observed earlier, and in general, excepting for the organs of the chest, the changes noted early in the epidemic remained the same.

A decided cyanosis of the face, neck and extremities was observed frequently, and commonly there was a distinct yellow tinge to the skin and conjunctivae. Dryness of the skeletal muscles was usual. The parietal pleura commonly was hyperemic and glistening, or slightly dull with loss of the normal smoothness and covered by a scanty layer of fibrin. Turbid brown fluid from a few cc to 300 cc was contained in each pleural cavity when the

inflammation had penetrated into these serous spaces. Changes in the pericardial sac consisted largely in an increase in the amount of yellow fluid normally contained. A true sero-fibrinous or suppurative pericarditis was not seen until late in the epidemic, and acute changes in the heart valves were not observed in any postmortem examination. The myocardium, and all the parenchymatous organs as well, presented a moderate or severe grade of cloudy swelling with commonly disseminated areas of acute fatty changes. Subserous petechial hemorrhages were common.

The parabranchial lymph glands constantly were enlarged, very soft, sometimes with small areas of necrosis. The lining of the trachea and the main bronchi was intensely hyperemic, and on the mucous membranes there was a thin milky, gray, brown, or sanguineous fluid, sometimes frothy. In the deeper portions of the respiratory tree the bronchioles were lined by an intensely red mucous membrane covered by a relatively thin, gray, exudate. The consolidation of the lungs was extensive. When removed from the chest they appeared voluminous, dark red or cyanotic, with no or very little exudate on the pleural surface, these membranes commonly being quite smooth and glistening. Underneath the pleura there were hemorrhages into the lung tissue from one to several centimeters extent as a rule, in addition to nodular consolidations which sometimes were so extensive as to form large confluent areas of firm tissue. Such extensive consolidation usually occurred in those patients that had survived several days, those in whom the lungs were extremely hemorrhagic dying relatively early in the disease. Several of the lobes were involved. From the surfaces of the lung tissue large quantities of blood and bloody fluid escaped, and there were firm areas of red tissue slightly granular and moist, with edematous and hemorrhagic lung tissue between. As the epidemic progressed gray nodular consolidations often confluent were noted. On the cut surface the tissue was distinctly granular and moist with a brown or brownish red exudate.

The yellow of the adrenal cortices was moderately diminished, or entirely absent and in one case there were several small subcapsular hemorrhages in each organ. As a rule the spleen was increased in size from one and one-half times to twice the normal, and the substance of the organ was soft and dark red. The biliary and mesenteric lymph nodes were constantly increased in size, the former much more so relatively than the latter, the substance moderately firm and red. Changes in the common bile duct, the portal vein, the splenic, and the upper portion of the superior mesenteric veins were not seen.

The liver was enlarged, its capsule tense and the tissue beneath mottled with disseminated areas of acute fatty changes and passive hyperemia. Focal necrosis was not recognized grossly. In the lining of the stomach there were multiple petechial hemorrhages.

In a limited number of cases in which the brain was examined there was found a moderate hyperemia of the pia-arachnoid, with dryness of the cerebral cortex. The spinal fluid of such patients usually without meningeal manifestations, commonly was turbid. In a limited number of examinations collection of a mucopurulent exudate was found in the sinuses of the face and middle ears.

As the epidemic progressed, complications commonly following pneumonia were found post mortem, including serofibrinous pleuritis, empyema of the chest, acute serofibrinous pericarditis, acute suppurative pericarditis and peritonitis. The character of the lung changes varied too in the later stages.

and it was in the last few days of the epidemic that gray consolidations of the lungs were noted, and in a few of these extensive softening of the lung tissue. In some, lung changes grossly corresponded with descriptions of acute suppurative interstitial pneumonia. Mention should be made of cases in which the lung changes were comparatively insignificant, but in which disease of some of the bony spaces of the head occurred and subsequently or coincidentally infection of the leptomeninges. In these the greatest interest centers in the bacteriologic examinations which are discussed later.

TABLE 3
PNEUMOCOCCI FROM LUNG CULTURES POSTMORTUM

Culture	Inulin Fermentation	Capsule		Bile Solubility	Amount of 24-Hour Blood-Agar Slant Inoculated into White Mouse	Dose Fatal in Hours	Type	Organism Recovered Pure from Mouse Heart Blood
		On Blood Agar	In Heart Blood of Mouse					
200	+	—	+	+	0.0231	19	II	+
207	+	—	+	+	0.00925	20	II	+
208	+	—	+	+	0.00925	20	II	+
210	+	—	+	+	0.0055	24	IV	+
21	+	—	+	+	0.0055	36	IV	+
221	+	—	+	+	0.0055	18	IV	+
226	+	—	+	+	0.0055	36	II	+
230	+	—	+	+	0.0055	24	II	+
231	+	—	+	+	0.0055	26	IV	+
251	+	—	+	+	0.0154	20	IV	—
259	+	—	+	+	0.0055	18	II	+
366	+	—	+	+	0.00185	34	II	+
273	+	—	+	+	0.0231	12	IV	+
296	+	—	+	+	0.0154	12	II	+
312	+	—	+	+	0.00925	20	II	+
316	+	—	+	+	0.0055	20	II	+
364	+	—	+	+	0.00185	30	IV	+
365	+	—	+	+	0.00155	30	II	+
266	+	—	+	+	0.0231	12	IV	+
369	+	—	+	+	0.00185	36	II	+
374	+	—	+	+	0.0055	30	II	+
404	+	—	+	+	0.0037	30	II	+
405	+	—	+	+	0.0037	30	II	+
425	+	—	+	+	0.0055	18	IV	+
434	+	—	+	+	0.0055	28	IV	+

+ positive; — negative.

Following the decline of the epidemic, deaths occurred in the hospital fairly often with diseases primarily pulmonary, the lung changes of which differed from those observed during the epidemic. Usually such deaths followed an illness in the hospital of more than one week. In the lungs of those dying within the first or second week there were red or reddish-brown nodular peribronchial consolidations irregularly scattered throughout one or more lobes. The bronchioles contained a white, viscid exudate, the lining membrane being hyperemic and swollen. As this process became moderately advanced, the lungs contained many soft, yellow, peribronchial areas of necrotic tissue from 1-3 mm. in diameter, contrasting sharply with the red, air-containing or compressed lung tissue. Removal of the exudate from these areas exposed shallow crater-like, peribronchial excavations in the tissue, without a definite limiting membrane, being covered by a yellow shaggy exudate. The tissue beneath the exudate was hyperemic or hemorrhagic. Lungs in which this peribronchial destruction of tissue was general were riddled by many abscesses.

of relatively small dimension without a definite limiting membrane. Such lobes sometimes were just masses of necrotic tissue held together by the supporting framework. Other lungs not as extensively diseased contained several or many scattered peribronchial abscesses of relatively small dimension having a thin pyogenic membrane.

Clinically, the patients with extensive destruction of the lung tissue expectorated huge quantities of yellow exudate and liquefied necrotic tissue, a phenomenon regarded by some as the emptying of an empyema through an eroded bronchus. The postmortem examination demonstrated clearly that the yellow expectoration came from the lung itself and not from the pleural cavity.

This acute suppurative interstitial pneumonia, however, was not the pneumonia that characterized the epidemic proper.

Microscopic examination of tissue from various places in diseased lungs disclosed irregularly distributed and developed inflammatory changes. In the alveolar spaces over small or wide areas corresponding with the gross changes, were red corpuscles in large numbers and leukocytes in the ratio usually in the circulating blood. Such areas were distributed irregularly. Other alveolar spaces either in areas of hemorrhage or elsewhere contained endothelial cells in addition to the leukocytes the number of endothelial cells increasing apparently with the progress of the lung lesion. Delicate strands of fibrin were present in such alveolar spaces, and also polymorphonuclear leukocytes which gradually approached and exceeded the endothelial cells in number. In later stages the alveolar spaces contained numerous polynuclear leukocytes, slightly less numerous endothelial cells, fewer red corpuscles, strands and masses of fibrin, cellular detritus, and amorphous particles of coagulated material.

In large confluent areas of consolidation the alveolar spaces regularly contained polynuclear leukocytes, endothelial cells, red corpuscles, and granular protein material as mentioned.

The bronchioles invariably contained many polynuclear leukocytes, endothelial cells, red corpuscles, fibrin, and other products of an acute inflammatory reaction.

CULTURES AT POSTMORTEM EXAMINATION

Of great importance are the results of bacteriologic examinations of diseased tissues, exudates, and fluids removed at the postmortem examinations. Laying aside for the moment all consideration of the predisposing factors of the disease, is it not fair to say that the power of blood stream invasion that an organism possesses determines in a large measure its pathogenicity for the host, and that the fairly constant isolation of the same organism from the blood stream of similarly diseased patients whether during life or soon after death determines with great probability the organism directly responsible for the disease or its fatal issue? Recognizing also in specific diseases that the causative organism frequently manifests a specific election for certain tissues, is it not likely that proper bacteriologic examinations of such diseased tissues in a sufficiently large number will determine the organism or organisms responsible for the local manifestation of the disease?

TABLE 4
PNEUMOCOCCI FROM MISCELLANEOUS CULTURES

Culture	Inulin Fermentation	Capsule in Heart Blood	Bile Solubility	Amount of 24-Hour Blood-Agar Slant Injected into White Mouse	Dose Fatal in Hours	Type	Organism Recovered Pure from Mouse Heart Blood	Source of Culture
2003	+	+	+	0.0154	20	II	+	Pericardial sac
2005	+	+	+	0.0154	12	II	+	Pericardial sac
2006	+	+	+	0.00925	18	I	+	Pericardial sac
2014	+	+	+	0.0154	50	I	+	Pericardial sac
2016	+	+	+	0.0154	48	II	+	Pericardial sac
2052	+	+	+	0.00925	17	I	+	Knee
2082	+	+	+	0.00925	32	IV	+	Knee
2075	+	+	+	0.00925	20	IV	+	Muscle abscess
2077	+	+	+	0.00925	32	IV	+	Muscle abscess
2010	+	+	+	0.0154	40	IV	+	Pleural fluid
2012	+	+	+	0.00925	28	IV	+	Pleural fluid
2013	+	+	+	0.00925	40	IV	+	Pleural fluid
2015	+	+	+	0.0154	72	IV	+	Pleural fluid

+ positive; — negative.

TABLE 5
PNEUMOCOCCI FROM SPINAL FLUID

Culture	Inulin Fermentation	Capsule in Heart Blood	Bile Solubility	Amount of 24-Hour Blood-Agar Slant Injected into White Mouse	Dose Fatal in Hours	Type	Organism Recovered Pure from Mouse Heart Blood
2004	+	+	+	0.00925	18	IV	+
2009	+	+	+	0.0154	30	II	+
2017	+	+	+	0.0154	18	I	+
2011	+	+	+	0.0154	50	I	+
2018	+	+	+	0.0154	42	I	+
2019	+	+	+	0.00925	18	II	+
2055	+	+	+	0.00925	36	II	+
2057	+	+	+	0.00925	18	II	+
2078	+	+	+	0.00925	15	IV	+
2086	+	+	+	0.00925	30	II	+
2039	+	+	+	0.00925	36	II	+
2042	+	+	+	0.00925	16	II	+
2043	+	+	+	0.00925	17	II	+

+ positive.

The last 3 cultures were obtained during life, the others after death.

Accordingly, heart blood was removed with a sterile pipet at each post-mortem examination, inoculated into dextrose broth, and after 24 hours' incubation examined for organisms by smears and by streaking out on plain blood-agar plates (0.5-0.7% acid to phenolphthalein). Cultures were made also from the consolidated lung tissues, bronchial exudates, pleural and pericardial fluids, and exudates in the sinuses and bony spaces of the head when these were found diseased. Thin fluids were inoculated into dextrose broth while thicker exudates were streaked out on plain blood agar. All of the latter and some of the former were controlled by making direct smears, stained according to Gram's method and counterstained with a dilute aqueous fuchsin solution.

During the epidemic there were 198 postmortem examinations, and at each cultures of the diseased tissues and fluids were made as stated. Many times, and this is particularly true in the early part of the epidemic, blood-agar plate cultures from the diseased lungs contained, purely fine, moist, transparent, slightly umbilicated, green colonies of gram-positive, lancet-shaped diplococci as such or in short chains. The bacillus of influenza was never found in pure culture and with other organisms in only six lung cultures. For convenience this gram-positive lancet-shaped diplococcus is called a pneumococcus for reasons to be presented later.

As to the incidence of the pneumococcus, the hemolytic streptococcus, and the bacillus of influenza, the first occurred in 144 lung cultures, the second in 58, the third in 6. These results were from a series of 198 postmortem examinations, 26 lung cultures of which were unsatisfactory, and in 5 of which no lung cultures were made.

The following table briefly summarizes the results.

Pure pneumococcus.....	89
Pneumococcus and hemolytic streptococcus.....	34
Pneumococcus and nonhemolytic streptococcus.....	13
Pneumococcus, hemolytic, and nonhemolytic streptococci..	3
Pneumococcus, influenza bacillus, and nonhemolytic streptococcus	2
Pneumococcus and influenza bacillus.....	2
Pneumococcus, influenza bacillus, and hemolytic streptococcus	1
Pure hemolytic streptococcus.....	19
Hemolytic streptococcus and influenza bacillus.....	1
Nonhemolytic streptococcus.....	3
Not cultured.....	5
Unsatisfactory	26

The importance of hemolytic streptococci in diseases of the respiratory passages is well recognized. During the early and middle stages of the epidemic this organism appeared sporadically, so to speak, but in the later stages when complications of pneumonia were observed its presence and frequency in the cultures increased. The incidence of the pneumococcus and the hemolytic streptococcus during the course of the epidemic is detailed graphically in chart 1, and from

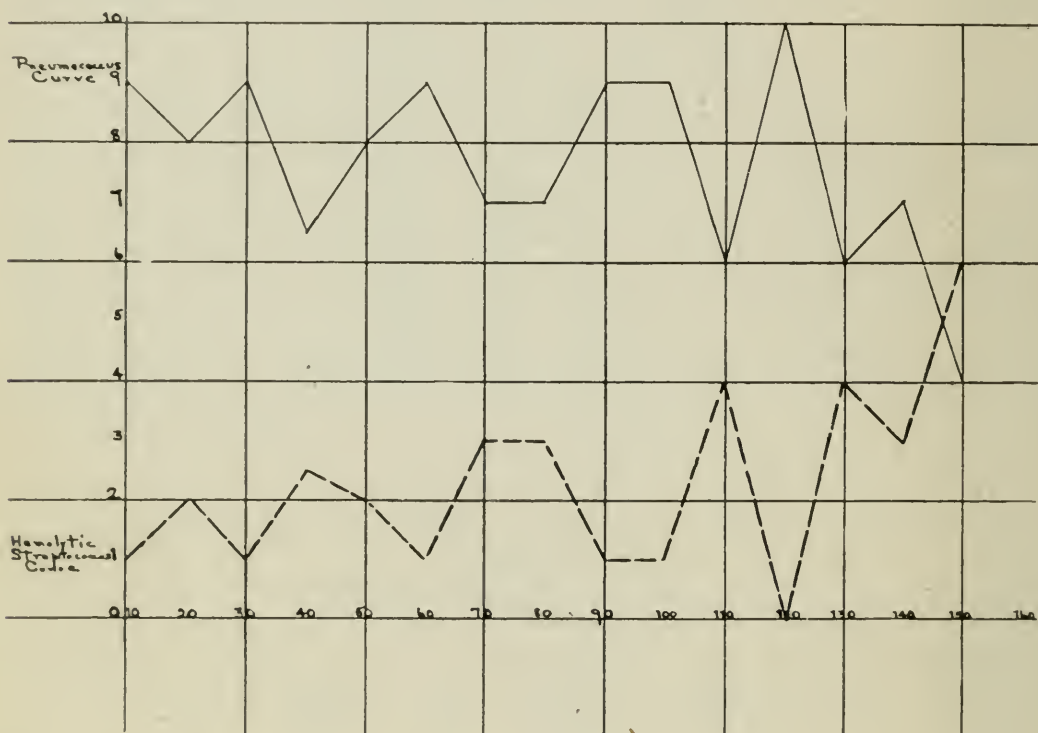


Chart 1.—The incidence curve of pneumococci and hemolytic streptococci in the postmortem bacteriologic studies during the epidemic. The curve was made by arranging in sequence all postmortem examinations with satisfactory bacteriologic studies from the beginning to the close of the epidemic and then in groups of ten noting the frequency of a predominating pneumococcus or hemolytic streptococcus. On the abscissae are plotted postmortem examinations in groups of ten. On the ordinate the number of times the pneumococcus or hemolytic streptococcus was the predominant organism in each group. Pneumococcus ———, Hemolytic Streptococcus — — — —.

this the importance of a careful study from the beginning to the close of the epidemic can be best appreciated. Where only a few chance examinations are made or where the study is limited to some portion of the curve an erroneous impression may be gained regarding the proper importance of the hemolytic streptococcus. Hemolytic streptococci appeared in 58 lung cultures of the 198 postmortem examinations as follows:

Pure hemolytic streptococcus.....	9
Hemolytic streptococcus and influenza bacillus.....	1
Hemolytic streptococcus and pneumococcus.....	19
Hemolytic streptococcus and pneumococcus with pneumococcus in heart blood	10
Hemolytic streptococcus and pneumococcus with hemolytic streptococcus in heart blood.....	8
Hemolytic streptococcus, pneumococcus, and influenza bacillus with pneumococcus in the heart blood.....	1
Hemolytic streptococcus with pneumococcus in the heart blood.....	2
Hemolytic streptococcus both in lungs and heart blood.....	7
Hemolytic streptococcus in lungs, heart blood and spinal fluid.....	1
	<hr/>
	58
Hemolytic streptococcus in heart blood with pneumococcus in lung.....	6
Hemolytic streptococcus and pneumococcus in heart blood with pneumococcus in lungs.....	1

The influenza bacillus was found in cultures with other organisms in 6 postmortem examinations distributed as follows:

Influenza bacillus with many pneumococci.....	2
Influenza bacillus with pneumococcus and nonhemolytic streptococcus.....	2
Influenza bacillus with pneumococcus and hemolytic streptococcus.....	1
Influenza bacillus with hemolytic streptococcus.....	1
	<hr/>
	6

The search for influenza bacilli in smears of the lung exudate and in all cultures was careful and thorough. The possibility of this organism being overgrown by other bacteria on the blood-agar plates is not probable, as surface inoculations uniformly were made so that any organism could be isolated or at least identified.

As control for the surface inoculation of plain blood agar in recovering the influenza bacillus from lung and other exudates as practiced during the epidemic, a large number of throat cultures were made after the epidemic comparing the blood agar surface inoculations with similar surface inoculations made on mediums especially favorable to the growth of the influenza bacillus. The tests were made by collecting posterior pharyngeal secretions on cotton applicators and inoculating in succession with the same cotton applicator the surface of a plain blood-agar plate, an Avery's selective medium plate,¹ and a "chocolate" medium plate. The latter medium was made by drawing horse blood into flasks containing sterile sodium citrate solution to make 1.5% final concentration. After the corpuscles had settled, the plasma was removed and sterile distilled water added up to the original volume of blood. Beef infusion agar with a reaction of plus 0.2% is heated to 90 C., and while at this temperature the laked blood is added up to 1-2%.

In these examinations the influenza bacillus was found as frequently on the blood-agar plates as on the other mediums.

¹ Jour. A. M. A., 1918, 71, p. 2050.

Cultures of the heart blood removed at the postmortem examination yielded most frequently a pneumococcus. The results obtained in 198 postmortem examinations are briefly:

Pneumococcus	53
Hemolytic streptococcus.....	23
Pneumococcus and hemolytic streptococcus.....	1
Nonhemolytic streptococcus.....	2
Negative	109
Not taken.....	10

198

Cultures of a limited number of spinal fluids removed at the postmortem examinations including three in which the leptomeninges were clouded by a thick yellow exudate accomplished the isolation of pneumococci in pure culture in eight, and bacterial examination of fluid removed by spinal puncture from patients in the hospital demonstrated pure culture of pneumococci in three others.

TABLE 6
PNEUMOCOCCI FROM BLOOD CULTURES DURING LIFE

Culture	Inulin Fermentation	Capsule		Bile Solubility	Amount of 24-Hour Blood-Agar Slant Injected into White Mouse	Dose Fatal in Hours	Type	Organism Recovered Pure from Mouse Heart Blood
		On Blood Agar	In Heart Blood of Mouse					
602	+	—	+	+	0.0231	18	II	+
605	+	—	+	+	0.0231	22	II	+
610	+	—	+	+	0.0231	18	IV	+
611	+	—	+	+	0.0231	30	IV	+
613	+	—	+	+	0.0231	18	IV	+
624	+	—	+	+	0.0231	18	IV	+
628	+	—	+	+	0.0231	14	II	+
631	+	—	+	+	0.0231	18	II	+
633	+	—	+	+	0.0231	18	IV	+
637	+	—	+	+	0.0231	15	II	+
640	+	—	+	+	0.0231	22	IV	+
643	+	—	+	+	0.0231	18	IV	+
644	+	—	+	+	0.0231	30	II	+
646	+	—	+	+	0.0231	18	II	+
658	+	—	+	+	0.0231	44	IV	+

+ positive; — negative.

Similarly, pure cultures of pneumococci were isolated from four pleural fluids removed postmortem and fluid taken from the pericardial sac at five other necropsies contained organisms morphologically identical in pure culture.

During the epidemic the left knee of a patient in the hospital became swollen and painful. Aspiration disclosed the presence of a

thick yellow exudate which culturally contained purely pneumococci. A postmortem examination later disclosed the presence of an extensive atypical suppurative interstitial pneumonia, culture of the lung tissue containing organisms identical with those recovered from the exudate in the knee joint. The exudate recovered from a gluteal abscess in another patient convalescent from pneumonia contained a pure culture of pneumococci.

In order to determine whether the gram-positive diplococcus isolated from the heart blood, lungs and other diseased tissues of the body was a pneumococcus according to recognized tests, the pure strains recovered were tested for their cultural characteristics on plain blood agar and in Hiss inulin serum water, for their tinctorial reaction

TABLE 7
PNEUMOCOCCI FROM ACCESSORY SINUSES AND EYE

Culture	Inulin Fermentation	Capsule in Heart Blood	Bile Solubility	Amount of 24-Hour Blood-Agar Slant Injected into White Mouse	Dose Fatal in Hours	Type	Organism Recovered Pure from Mouse Heart Blood	Source of Culture
2022	+	+	+	0.00925	36	IV	+	Conjunctiva
2023	+	+	+	0.00925	28	IV	+	Ear
2029	+	+	+	0.00925	40	II	+	Ear
2030	+	+	+	0.00925	18	IV	+	Antrum of Highmore
2056	+	+	+	0.00925	32	IV	+	Mastoid
2080	+	+	+	0.00925	12	IV	+	Rt. frontal, postmortem
2084	+	+	+	0.00925	30	IV	+	Sphenoid sinus, postm.
2087	+	+	+	0.00925	30	II	+	Mastoid

+ positive; — negative.

and morphology by staining according to Gram's method, and for the presence of a capsule by staining according to the Hiss technic. Measured fractional quantities of a 24-hour blood-agar slant were inoculated intraperitoneally into white mice to determine (1) an approximation of the virulence the various strains might have, and (2) to test further the morphology and type of pneumococcus in the mouse. The approximate virulence for white mice, the presence of a capsule, the reaction in inulin, the bile solubility and the type are given in table 2 for a few strains recovered from the heart blood post mortem. The same features are given for a few isolated from the lungs, from various sources, and from spinal fluids in tables 3, 4 and 5, respectively. Without exception, practically all strains not listed reacted culturally the same and appeared identical morphologically.

BLOOD CULTURE DURING LIFE

Having found a pneumococcus in the heart blood at postmortem examination of patients dying with pneumonia during the epidemic, the next step was to attempt its isolation from the blood stream of patients in the hospital. The usual inoculation of a few cubic centimeters of whole blood into broth or other medium did not seem as good a method as that of taking larger quantities of blood and inoculating the centrifugalized sediment into a suitable medium. The method therefore used is as follows: About 10 c c of blood was drawn aseptically into $\frac{1}{2}$ c c of 15% sodium citrate in a large sterile centrifuge tube and thoroughly mixed. To this 4 or 5 volumes of sterile distilled water were added to lase the red corpuscles, and the liquid then centrifuged in a large centrifuge at about 2,000-2,500 r. p. m. for 20-30 minutes. The supernatant liquid was drawn off with a sterile pipet, and the sediment inoculated into melted blood agar and plated in sterile Petri dishes. After 24-36 hours' incubation, the plates were examined for colonies of bacteria.

During the first few days of the epidemic, cultures were made from the blood of patients with pneumonia, and of the number taken, seventeen contained small green colonies of pneumococci. At the beginning, the total number of cultures on epidemic patients was not separated from others taken in the hospital, so that in this series, the percentage of positive results cannot be determined accurately. These first attempts, however, demonstrated clearly that invasion of the blood stream by a pneumococcus was present, and at once a systematic investigation was started. Using the method outlined, and without more than one attempt in all but 3 or 4, blood cultures were made on 90 patients in the hospital wards. Of this number 45 were positive, the colonies on the blood-agar plates without a single exception being small, moist, surrounded by a distinct green halo and containing organisms morphologically pneumococci.

The total number of strains obtained pure by blood cultures is 62, and based on the series of 90 blood cultures taken, in which 45 proved positive, the organism was isolated in 50% of the patients tested. That this does not represent the highest percentage possible is understood by the statement that with the exception of three or four patients, only one culture was made with each patient. Of all the positive blood cultures obtained from patients in the hospital with the epidemic disease not one contained organisms other than the pneumococcus.

Additional cultural and morphological data for a few of these strains are contained in table 8.

TABLE 8
PNEUMOCOCCI IN PLEURAL EXUDATES

Culture	Inulin Fermentation	Capsule in Heart Blood	Bile Solubility	Amount of 24-Hour Blood-Agar Slant Injected into White Mouse	Dose Fatal in Hours	Type	Organism Recovered Pure from Mouse Heart Blood
2024	+	+	+	0.00925	36	II	+
2028	+	+	+	0.00925	28	IV	+
2034	+	+	+	0.00925	24	II	+
2046	+	+	+	0.00925	17	IV	+
2048	+	+	+	0.00925	15	II	+
2051	+	+	+	0.00925	32	I	+
2054	+	+	+	0.00925	17	II	+
2060	+	+	+	0.00925	15	IV	+
2063	+	+	+	0.00925	15	I	+
2066	+	+	+	0.00925	12	IV	+
2069	+	+	+	0.00925	12	II	+
2085	+	+	+	0.00925	30	II	+

+ positive.

CULTURES OF EXUDATES RECOVERED FROM SINUSES AND BONY SPACES OF THE HEAD

Invasion of the bony spaces of the head is not uncommon in severe infections of the nasopharynx. It was not surprising, therefore, to find such complications in patients during the epidemic. In such, the nasopharynx or upper respiratory passages had been especially if not solely involved. Acute suppurative otitis media was the most common severe upper respiratory complication. Many of these diseased middle ears were drained without bacteriologic examination so that the reports of such with bacteriologic studies are few. Others examined bacteriologically several days after artificial drainage contained such a variety of organisms that the initial invader could not be determined. The exudate escaping with paracentesis of the ear-drum in three patients was cultured on plain blood-agar plates, and the growth after 24 hours' incubation was purely fine green colonies of pneumococci. Other organisms were not present.

In two patients acute suppurative mastoiditis occurred and from the exudate pure cultures of the same organism were recovered. In two other patients involvement of the antrum of Highmore followed the nasopharyngeal infection and from the exudate a pure culture of pneumococci was isolated. And cultures of exudates discovered in the bony sinuses of the head at the postmortem examinations again gave

the organism in pure culture. The conjunctiva in one patient became infected and a pure culture of pneumococci was isolated from the exudate. The results of morphological, cultural and virulence tests of the cultures isolated from the sinuses of the ear and the eye are given in table 7.

CULTURES OF PLEURAL EXUDATES

The collection of a purulent exudate in the chest as a complication of pneumonia is common. The acute stages of pneumonia in a large number of patients admitted during the epidemic was followed or complicated by empyema. This is not the place to discuss at length empyema following closely in the wake of the epidemic proper, but it does seem important to give here the results of cultures of the fluid aspirated from the chest of patients in whom a purulent exudate formed. The exudate was removed aseptically with needle and syringe, and cultured promptly on plain blood-agar plates. The aspirated fluid from 74 empyemas was examined with the following results:

Pure pneumococcus	46
Pneumococcus and hemolytic streptococcus.	5
Pure hemolytic streptococcus.....	22
Pure nonhemolytic streptococcus.....	1
	—
Total	74

This table shows that a pneumococcus was recovered in pure culture in 62% of the fluids examined. These strains of pneumococci, too, were identified more closely by further study, the results of which are shown in table 8.

VIRULENCE EXPERIMENTS

During the sudden rush of work coming necessarily with an epidemic, certain general methods must be followed in order to gather as much material as possible, to be studied later. From many sources pure strains of pneumococci were isolated and kept on artificial mediums until their cultural, morphologic, and virulence properties could be systematically determined. Realizing that the virulence of these organisms might diminish with cultivation on artificial mediums the determination of virulence was taken up with least possible delay. With some cultures, however, the determination necessarily was delayed until 4 or 5 weeks after their first isolation.

The white mouse was chosen for this purpose, as both virulence determination and typing of the pneumococcus could be done in one animal. Rabbits and guinea-pigs were used also. In order to approximate a standard unit for inoculation, all transfer cultures were inoculated on plain blood agar slanted in culture tubes of 16 by 150 mm. dimension, approximately the same surface area being inoculated with the same quantity each time. The growth after 24 hours' incubation was suspended in measured quantities of normal salt solution, the surface of the agar being brushed off with a camel's hair brush. Further standardization was accomplished by establishing a unit loop made of medium sized platinum wire. The number of loops in 1 c c of salt solution was found to be 108. Taking up the 24 hours' growth of bacteria on a blood-agar slant in a definite number of cubic centimeters of salt solution, and knowing the number of loops contained in one c c the amount of a 24-hour blood-agar slant culture inoculated can be expressed in a decimal fraction.

All the strains isolated from the heart blood, from the lungs, and from various other foci of the body postmortem, from the circulating blood, the middle ear, the maxillary sinuses, the pleural cavity, and from other places were tested for their virulence in white mice.

In order to carry out virulence tests on such a large number of cultures it was necessary to determine approximately the virulence of a few strains in white mice, and having determined a minimal dose in a small group to try this same amount in a large number. For the strains isolated from the blood stream and for those isolated from the heart blood postmortem 0.0231 of 24-hour blood-agar culture killed constantly, some of the cultures causing death sooner than others. The number of hours required to kill a white mouse of average size is given for some of the heart blood cultures in chart 4, and for certain blood cultures in chart 8.

When the virulence of the strains isolated from the lungs post-mortem and of those isolated from various exudates obtained during life was examined, it was found to exceed that determined for the heart blood and the blood culture strains. Consequently the dosage was diminished at first to 0.0154 of 24-hour blood-agar slant culture, and when this was found to be rapidly fatal it was diminished to 0.00925 of a culture, then to 0.00555, and finally as low as 0.00185. The smallest dose tested in a few cultures was found to be fatal to white mice in from 30-36 hours. The amount of 24-hour blood-agar culture inoculated into a white mouse and the time required to kill is

given for some of the lung cultures in table 3, for those recovered from the spinal fluid postmortem and during life in table 5, and for those isolated from pleural and the pericardial fluids and from embolic foci postmortem and during life in table 4, for those from pleural exudates in table 8, for those from infected sinuses of the head, mastoid, and eye in table 7, and for those from the throat in table 3.

In all of these virulence experiments the organism practically without exception was recovered from the heart blood of the mouse in pure culture. The peritoneal exudate was used in determining the type of pneumococcus with the antipneumococcus agglutinating serums.

To determine the virulence of some of the strains in rabbits and guinea-pigs further tests were made as follows:

1.—Rabbit, 1,625 gm. injected intravenously with 1-18 hour blood-agar slant of blood culture No. 655. Dead after 16 hours. Pure culture of organism recovered from heart blood and peritoneal exudate.

2.—Guinea-pig, 285 gm., injected intraperitoneally with 1-22 hour blood-agar slant culture of blood culture No. 655. Dead after 13 hours. Pure culture of organism recovered from the heart blood and peritoneal exudate.

3.—0.25 of 1-24 blood-agar slant culture of designated strains was inoculated intraperitoneally as follows: Guinea-pig, 345 gm., blood culture No. 606, dead after 60 hours. Guinea-pig, 297 gm., blood culture No. 616, dead after 39 hours. Guinea-pig, 325 gm., blood culture No. 651, dead after 39 hours. Guinea-pig, 325 gm., blood culture No. 658, dead after 47 hours. Guinea-pig, 288 gm., blood culture No. 604, lived. Guinea-pig, 390 gm., blood culture No. 621, lived. Guinea-pig, 326 gm., blood culture No. 608, lived. Guinea-pig, 277 gm., blood culture No. 611, lived. Guinea-pig, 334 gm., blood culture No. 639, lived. Guinea-pig, 298 gm., blood culture No. 660, lived. Organism recovered in pure culture from the heart blood and peritoneal exudate of all the animals that died.

4. 1-18 hour blood-agar slant culture of heart blood culture No. 1000 washed off in 4 cc of salt solution. Guinea-pig, 245 gm., received $\frac{1}{2}$ cc of bacterial suspension intraperitoneally. Lived. Guinea-pig, 345 gm., received 1 cc of bacterial suspension intraperitoneally. Lived. Guinea-pig, 330 gm., received $2\frac{1}{2}$ cc of bacterial suspension intraperitoneally. Dead after 45 hours. Organism recovered in pure culture in the heart blood and in the peritoneal exudate.

5.—1-18 hour blood-agar slant culture of heart blood culture No. 1004 washed off in 4 cc of salt solution. Guinea-pig, 230 gm., received $\frac{1}{2}$ cc of bacterial suspension intraperitoneally, dead after 17 hours. Guinea-pig, 234 gm., received 1 cc of bacterial suspension intraperitoneally, dead after 19 hours. Guinea-pig, 255 gm., received $2\frac{1}{2}$ cc of bacterial suspension intraperitoneally, dead after 20 hours. Pure culture of organism isolated from the heart blood and peritoneal exudate of each animal.

6.—Inoculated 0.5 of 1-24 hour blood-agar slant culture of designated strains intraperitoneally into guinea-pigs as follows: Guinea-pig, 308 gm., heart blood culture No. 1036, dead after $44\frac{1}{2}$ hours. Guinea-pig, 325 gm., heart blood culture No. 1040, dead after 10 hours. Guinea-pig, 290 gm., heart blood culture No. 1036, lived. Guinea-pig, 269 gm., heart blood culture No. 1037, dead after 68 hours. Guinea-pig, 332 gm., heart blood culture No. 1033, dead after 6 days. Organism recovered in pure culture from heart blood and peritoneal exudate.

7.—1-24 hour blood-agar slant culture of lung culture No. 331 suspended in 5 cc of normal salt solution. Guinea-pig, 303 gm., 0.1 culture intraperitoneally, dead after 47 hours. Guinea-pig, 244 gm., 0.2 culture intraperitoneally, dead after 19 hours. Guinea-pig, 264 gm., 0.3 culture intraperitoneally, dead after 33 hours. Guinea-pig, 265 gm., 0.4 culture intraperitoneally, dead after $25\frac{1}{2}$ hours. Organism recovered in pure culture from heart blood and peritoneal exudate of all animals.

8.—1-24 hour blood-agar slant culture of lung culture No. 350 suspended in 5 cc of normal salt solution. Rabbit, 1,320 gm., 0.1 culture intravenously, dead after 31 hours. Rabbit, 1,580 gm., 0.2 culture intravenously, dead after 37 hours. Rabbit, 1,600 gm., 0.3 culture intravenously, dead after 20 hours. Rabbit, 1,590 gm., 0.4 culture intravenously, dead after 15 hours. Organism recovered in pure culture from heart blood of each animal.

9.—Rabbit, 2,222 gm., 0.1-24 hour culture of lung culture No. 347 intravenously, dead after $6\frac{3}{4}$ days. Organism recovered pure from the heart blood. Rabbit, 2,360 gm., 0.1-24 hour culture of lung culture No. 306 intravenously, lived, lost 200 gm. in 5 days. Rabbit, 2,165 gm., 0.1-24 hour culture of lung culture No. 340 intravenously, lived, lost 370 gm. in 10 days.

10.—Guinea pig, 274 gm., 0.1-24 hour culture of lung culture No. 347 intraperitoneally, dead after 60 hours. Guinea-pig, 290 gm., 0.1-24 hour culture of lung culture No. 306, intraperitoneally, dead after 60 hours. Guinea-pig, 297 gm., 0.1-24 hour culture of lung culture No. 340 intraperitoneally, dead after 60 hours. Pure cultures of the organism were isolated from heart blood and peritoneal cavities of all the animals.

11.—Quantitative virulence experiment in white mice. Lung culture No. 347. White mouse, 0.00925 24 hour culture, dead after 17 hours. White mouse, 0.00925 24 hour culture, dead after 19½ hours. White mouse, 0.000925 24 hour culture, dead after 19 hours. White mouse, 0.000925 24 hour culture, dead after 22 hours. White mouse, 0.0000925 24 hour culture, dead after 22 hours. White mouse, 0.0000925 24 hour culture, dead after 28 hours. Pure cultures of the organism were isolated from heart blood and peritoneal cavities of all the animals.

12.—Quantitative virulence experiment in white mice. Lung culture No. 306. White mouse, 0.00925 24 hour culture, dead after 13½ hours. White mouse, 0.00925 24 hour culture, dead after 13½ hours. White mouse, 0.000925 24 hour culture, dead after 14½ hours. White mouse, 0.000925 24 hour culture, dead after 14½ hours. White mouse, 0.0000925 24 hour culture, dead after 14½ hours. White mouse, 0.0000925 24 hour culture, dead after 15 hours. Pure cultures of the organism were isolated from heart blood and peritoneal cavities of all the animals.

13.—Quantitative virulence experiment in white mice. Lung culture No. 340. White mouse, 0.00925, 24 hour culture, dead after 14½ hours. White mouse, 0.00925, 24 hour culture, dead after 27½ hours. White mouse, 0.000925, 24 hour culture, dead after 26½ hours. White mouse, 0.000925, 24 hour culture, dead after 26½ hours. White mouse, 0.0000925, 24 hour culture, dead after 14½ hours. White mouse, 0.0000925, 24 hour culture, dead after 27½ hours. Pure cultures of the organism isolated from heart blood and peritoneal cavities of all the animals.

14.—0.1 of 1-24 hour blood agar slant culture of designated lung strains was inoculated intraperitoneally as follows: Guinea-pig, 390 gm. lung culture No. 310, lived, lost 35 grams in 2 days. Guinea-pig, 293 gm., lung culture No. 305, dead after 68 hours. Guinea-pig, 263 gm., lung culture No. 344, dead after 6 days. Guinea-pig, 413 gm., lung culture No. 358, dead after 9 days. Guinea-pig, 389 gm., lung culture No. 365, lived, no effect. Guinea-pig, 359 gm., lung culture No. 369, lived, lost 93 grams in 8 days. Guinea-pig, 274 gm., lung culture No. 380, lost 20 gm. in 6 days. Guinea-pig, 376 gm., lung culture No. 376, dead after 52 hours. Guinea-pig, 396 gm., lung culture No. 398, lived, lost 73 grams in 8 days. Guinea-pig, 365 gm., lung culture No. 406, dead after 50 hours. Organism recovered in pure culture from the heart blood and peritoneal exudate of all the animals that died.

15.—0.1 of 1-24 hour blood-agar slant culture of the following designated strains injected intraperitoneally as follows: Guinea-pig, 340 gm., spinal fluid culture No. 3042, dead after 28½ hours. Guinea-pig, 362 gm., spinal fluid culture No. 2083, lived, lost 51 grams in 4 days. Guinea-pig, 430 gm., pleural fluid culture No. 2070, lived, lost 96 grams in 4 days. Guinea-pig, 433 gm., mastoid culture No. 2056, lived, lost 51 grams in 4 days. Guinea-pig, 383 gm., mastoid culture No. 2087, dead after 14 hours. Guinea-pig, 324 gm., pleural fluid culture No. 2089, dead after 24 hours. Guinea-pig, 355 gm., pleural fluid culture No. 2051, lost 43 gm. in 4 days. Pure culture of organism recovered from peritoneal cavity of 11 the animals that died and from the heart blood of these same animals excepting guinea-pig (No. 2087).

16.—0.1 of 1-24 hour blood-agar slant culture of designated strains injected intravenously as follows: Rabbit, 1549 gm., spinal fluid culture No. 2042, lived and lost 120 gm. in 4 days. Rabbit, 1751 gm., spinal fluid culture No. 2083, lived and lost no weight. Rabbit, 1439 gm., mastoid culture No. 2056, lived and lost 140 gm. in 2 days. Rabbit, 1569 gm., mastoid culture No. 2087, lived and lost 150 gm. in 2 days. Rabbit, 1792 gm., pleural fluid culture No. 2070, dead after 24 hours.

These virulence tests have demonstrated a highly lethal property for practically all cultures in animals, a small part of a 24-hour blood-agar slant culture being regularly fatal.

Throat cultures have proven to be the least virulent. The detection of virulent strains of pneumococci is impossible simply by judging from the appearance of the colony, and in transferring many colonies nonvirulent strains may be isolated as readily as the virulent, depending, of course, on the frequency of one or the other, and the element of chance. Our throat cultures, further, were grown on artificial mediums for some time before virulence tests could be made. However, it is safe to say that the upper respiratory passages were invaded by virulent pneumococci inasmuch as a number of definitely virulent strains were isolated from the throat, while organisms quite as virulent as those recovered from the lungs, were isolated from the sinuses of the head and from the middle ear.

The inoculations in white mice indicate high virulence. The smallest dilution, representing 0.0000925 of a 24-hour blood-agar slant culture, killed in a little more than 12-24 hours. In order to determine how many organisms this dilution represents three 24-hour blood-agar slant cultures were taken up, respectively, in 5 cc of normal salt solution and dilutions made as before:

(a) Five loops of bacterial suspension in 1 cc sterile distilled water — 5/540 or 0.00925 per cc.

(b) 0.5 of (a) in 4.5 cc sterile distilled water — 5/5,400 or 0.000925 per cc.

(c) 0.5 cc of (b) in 4.5 cc distilled water.

One cc of dilution (c) representing 0.0000924 of a culture was centrifuged in a clean sterile centrifuge tube at 2,000 to 2,500 r. p. m. for 1 hour. The supernatant liquid was removed with a capillary pipet and the last drops containing the bacteria transferred to a slide, allowed to dry, fixed, stained, and the pneumococci counted. The average count of these three estimations was 200 organisms, there being a variation of 35 between the maximum and the minimum counts. It seemed theoretically possible at least, from these determinations to increase the final dilution given above by 10 and have a suspension containing roughly 20 organisms, to the cc., or to increase that same dilution by 100 and have a suspension containing about 2 organisms in a cc. Accordingly, 24-hour blood-agar slant growths of lung cultures 306 and 347 were prepared. The growth of each one was taken up in 5 cc of normal salt solution and dilutions then made with sterile distilled water as follows:

(a) Fifteen loops of bacterial suspension in 3 cc sterile distilled water — 5/540 or 0.00925 culture per cc.

(b) 0.5 cc of (a) in 4.5 cc sterile distilled water — 5/5400 or 0.000925 per cc.

(c) 0.5 cc of (b) in 4.5 cc sterile distilled water — 5/54000 or 0.0000925 per cc.

(d) 0.5 cc (c) in 4.5 cc sterile distilled water — 5/540000 or 0.00000925 culture per cc.

(e) 0.5 cc of (d) in 4.5 cc sterile distilled water — 5/5400000 or 0.000000925 culture per cc.

Considered on the basis of the estimations which gave approximately 200 organisms in 0.0000925 cultures as represented by dilution (c), then dilution (d) would contain about 20 organisms per cc, and dilution (e) only a few; 10 white mice were selected for each series of culture dilutions, and in groups of two they were inoculated as follows:

Lung culture No. 306.—White mouse, 0.00925 24 hour culture, dead after 12 hours. White mouse, 0.00925 24 hour culture, dead after 12 hours. White mouse, 0.000925 24 hour culture, dead after 14 hours. White mouse, 0.000925 24 hour culture, dead after 15 hours. White mouse, 0.0000925 24 hour culture, dead after 32 hours. White mouse, 0.0000925 24 hour culture, dead after 24 hours. White mouse, 0.00000925 24 hour culture, dead after 30 hours. White mouse, 0.00000925 24 hour culture, lived. White mouse, 0.00000925 24 hour culture, lived. White mouse, 0.000000925 24 hour culture, lived.

Lung culture No. 347.—White mouse, 0.00925 24 hour culture, dead after 12 hours. White mouse, 0.00925 24 hour culture, dead after 12 hours. White mouse, 0.000925 24 hour culture, dead after 12 hours. White mouse, 0.000925 24 hour culture, dead after 15 hours. White mouse, 0.0000925 24 hour culture, dead after 12 hours. White mouse, 0.0000925 24 hour culture, dead after 12 hours. White mouse, 0.00000925 24 hour culture, dead after 12 hours. White mouse, 0.00000925 24 hour culture, dead after 15 hours. White mouse, 0.000000925 24 hour culture, dead after 17 hours. White mouse, 0.000000925 hour culture, dead after 17 hours. Pure cultures of the organism were isolated from heart blood and peritoneal cavities of all the animals.

After the inoculation of each dilution the glass syringe and needle used were placed in boiling water so that there could be no transfer of organisms in succeeding inoculations, and three control mice after each had received 1 cc of sterile distilled water intraperitoneally were placed in the cage with the inoculated mice. To control further the amount of bacterial suspension

inoculated, 1 cc of dilution (d) of each culture representing 0.00000925 culture was centrifuged in a clean centrifuge tube for 1 hour at 2,000 r. p. m., and in the stained sediment no pneumococci was found. Where only a few bacteria are contained in a liquid it is quite within the limits of technical error to fail in finding them, but at any rate the experiment indicates that the number of organisms suspended in the tested dilution was very small. To find that all of the mice inoculated with dilutions of culture 347 were dead within 17 hours is rather startling, and were the experiment not controlled carefully one might think that the results were falsified in some way. However, the results obtained with culture 306 as detailed, support the results obtained with culture 347, and pure cultures of pneumococci were isolated from the heart blood of all the animals that died. All of the control mice lived.

Following the lung cultures in the degree of virulence are the strains recovered from the heart blood at postmortem and from blood cultures during life. Why the strains isolated from the lungs and other sources of the body should possess the greatest virulence is not clear.

TABLE 9
THE RESULTS OF TYPE DETERMINATIONS OF PNEUMOCOCCI

Souree	No. of Cultures Tested	Type I	Type II	Type III	Type IV
Throat.....	8	..	3	..	5
Lungs at postmortem.....	201	12	109	2	78
Heart blood.....	67	5	45	..	17
Spinal fluid.....	12	3	7	..	2
Miscellaneous.....	13	3	3	..	7
Blood cultures.....	57	1	35	..	21
Exudates from:					
I Sinuses of head.....	8	..	2	..	6
II Chest.....	38	6	15	..	17
Total.....	404	30	219	2	153
Percentages.....		7.42%	54.20%	0.49%	37.88%

An organism has been described which, according to standard methods of recognition, is a pneumococcus. All the investigations of the epidemic demonstrate clearly that if the organism isolated from the sources mentioned be considered a pneumococcus, it must possess qualities which make it distinctive from other pneumococci, both in the power of invading tissues and in the lesion it produces in the host. All experimental work with this pneumococcus supports the conclusion that the strains of pneumococci recovered during the epidemic possess a high virulence. In this respect these organisms demonstrate a feature which makes them distinctive from other pneumococci. Such distinctive quality further explains why these pneumococci were able to produce an epidemic disease.

The results of the type determinations are given in table 9.

CONTROL INVESTIGATIONS

In order to have comparative data for the many throat examinations made on patients during the epidemic, throat cultures were taken on plain blood-agar plates from 50 of the prisoners. This group of men had been in the camp since last spring (1918), and not one of them had been ill with the

TABLE 10

THROAT CULTURES FROM PRISONERS—ORGANISMS OTHER THAN PNEUMOCOCCUS

Cultures	Organisms Found
1	Streptococci, nonhemolytic
2	Streptococci, nonhemolytic, staphylococcus
3	Streptococci, hemolytic
10	Streptococci, nonhemolytic
12	Streptococci, hemolytic
13	Streptococci, nonhemolytic, staphylococcus
14	Streptococci, hemolytic, staphylococcus
17	Streptococci, nonhemolytic
22	Streptococci, hemolytic
26	Streptococci, nonhemolytic, staphylococcus and <i>Micrococcus catarrhalis</i>
27	Streptococci, hemolytic, staphylococcus
28	Streptococci, hemolytic
29	Streptococci, nonhemolytic, staphylococcus
30	Streptococci, nonhemolytic, staphylococcus and <i>Micrococcus catarrhalis</i>
31	Streptococci, hemolytic, staphylococcus
32	Streptococci, nonhemolytic, staphylococcus
33	Streptococci, hemolytic, staphylococcus
36	Streptococci, hemolytic, staphylococcus
37	Streptococci, nonhemolytic, staphylococcus
38	Streptococci, hemolytic, staphylococcus
42	Streptococci, hemolytic, staphylococcus
43	Streptococci, hemolytic, staphylococcus
44	Streptococci, hemolytic, staphylococcus
47	Streptococci, hemolytic, staphylococcus

TABLE 11

THROAT CULTURES FROM PRISONERS—PROPERTIES OF PNEUMOCOCCI ISOLATED

Culture	Inulin Fermentation	Capsule		Bile Solubility	Amount of 24-Hour Blood-Agar Slant Injected into White Mouse	Dose Fatal in Hours	Type	Organism Recovered Pure from Mouse Heart Blood
		On Blood Agar	In Heart Blood of Mouse					
4	+	—	—	..	0.25	—	IV	—
6	—	—	—	..	0.25	+		
7	—	—	—	+	1.0	12		
8	+	—	—	..	0.5	—		
9	—	—	—	..	0.5	—	IV	+
15	—	—	—	+	1.0	12		
11	—	—	—	..	0.5	—		
16	+	—	—	..	0.5	—		
18	—	—	—	..	0.5	—	IV	—
19	+	—	—	..	0.25	—		
20	+	—	—	..	0.25	—		
21	+	—	—	..	0.5	—		
23	—	—	—	+	1.0	12	IV	—
24	+	—	—	..	0.5	—		
25	+	—	—	+	0.5	31		
34	—	+	+	+	1.0	12		
35	+	—	—	..	0.25	—	IV	—
39	+	—	—	..	0.25	—		
40	—	—	+	..	0.5	14		
41	+	—	—	+	0.5	14		
45	—	—	—	..	0.5	—	IV	—
48	+	—	—	+	0.5	14		
49	—	—	—	..	0.5	—		
50	—	—	—	..	0.5	—		

+ positive; — negative.

epidemic disease. The cultures were made with sterile cotton applicators, the secretions collected from the posterior pharynx being inoculated in surface smears on plain blood agar plates.

Table 10 records the bacterial flora in the 24 throat cultures in which pneumococci were not found. Chart 12 details certain morphological, cultural and virulence tests of a pneumococci isolated in 25 other throat cultures. One of the throat cultures was unsatisfactory. White mice were inoculated intraperitoneally with one-quarter, one-half, and entire 24-hour blood-agar slant cultures of the isolated strains of pneumococci. Eight of these strains of which one-half and entire 24-hour cultures had been inoculated were fatal to the mice. Therefore it was necessary to try a smaller quantity. One-quarter of a 24-hour growth of these strains was then inoculated. Six of the eight mice died, but in only three was the organism recovered from the heart blood. Given to guinea-pigs in the same quantity, these three strains had no effect.

The result of this survey indicates that with the exception of three strains of pneumococci, the pneumococci recovered in throat cultures of the prisoners possessed no unusual pathogenic property.

SUMMARY

An epidemic of unusual virulence swept with great rapidity through several organizations in Camp Grant between Sept. 21, 1918, and Oct. 18, 1918 (approximately). During this time 9,037 patients were admitted to the base hospital, representing about one-fourth the strength of the camp, and of them, 26% developed pneumonia. About 11% of the total admissions, or 43% of the total pneumonia patients died.

From the throat cultures of the early admissions pneumococci were recovered with remarkable constancy, influenza bacilli were found exceptionally.

Postmortem examination of patients dying during the epidemic demonstrated regularly a massive bronchopneumonia.

In cultures of the diseased lungs, heart blood, exudates, fluids, and other diseased foci of the body, pneumococci were the predominating organisms.

Blood cultures of 90 patients in the hospital with pneumonia of the epidemic disease were positive in 50%, pneumococci being the only organisms recovered.

Pneumococci in pure cultures were recovered from infected sinuses of the head, the middle ear, conjunctival and empyema exudates.

Inoculation of animals with pneumococci isolated during the epidemic demonstrated a high virulence of these organisms.

Control throat cultures made of prisoners in the camp escaping the epidemic disease contained practically no pathogenic organisms.

EFFECT OF FOREIGN PROTEIN ON THE KIDNEY

E. T. BELL AND T. B. HARTZELL

From the Department of Pathology, University of Minnesota, and the National Dental Research Association

It is known that some persons develop symptoms of anaphylactic character after eating certain foods, of which shellfish, egg-white, and certain fruits are familiar examples. It is believed by many that asthma, urticaria and hay-fever have their origin largely in a condition of hypersensitiveness to special foreign proteins. The symptoms produced when a foreign protein is introduced into a sensitized animal are usually referred to a hypothetical substance—"anaphylatoxin"—formed by the action of antibody on antigen. There are reports in the literature to the effect that anatomic changes in the tissues occur in acute anaphylaxis, and Longcope¹ has advanced the theory that repeated anaphylactic shock may be responsible for such chronic diseases as nephritis. By a series of animal experiments Longcope believes that he has shown that repeated nonfatal poisoning with foreign protein is responsible for some cases of chronic nephritis, cirrhosis of the liver, and chronic myocarditis. Our interest in the etiology of chronic nephritis led us to repeat some of his experiments.

ANATOMIC CHANGES IN ACUTE ANAPHYLAXIS

Gay and Southard² described hemorrhages and areas of fatty degeneration in guinea-pigs that died in acute anaphylactic shock and in those killed subsequent to severe shock. The hemorrhages were widely distributed, but were most commonly found in the mucosa of the stomach and in the lungs. Areas of fatty degeneration were found in capillary endothelium, in the mucosa of the stomach, and in the cardiac and voluntary muscle. The hemorrhages were interpreted as the result of the degeneration of the capillary endothelium. But Anderson and Rosenau³ in repeating this work could find no evidence

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¹ *Jour. Exper. Med.*, 1913, 18, p. 678; 1915, 22, p. 793; *Trans. Assn. Am. Phys.*, 1914, 29, p. 49.

² *Jour. Med. Research*, 1907, 16, p. 143; 1908, 18, p. 17.

³ *Jour. Med. Research*, 1908, 19, p. 57.

of fatty degeneration. They did not find the hemorrhages so constantly and seem inclined to interpret them, when present, as due to the violent struggling of the animal during shock rather than to the action of the protein.

It is known also that fatty degeneration does not occur suddenly. Usually several days are required for a cell to become definitely fatty, and very little fat accumulates earlier than 48 hours after the injury. It is inconceivable that this change could occur within a few minutes or even within a few hours. Again the normal variations in the fat content of voluntary muscle fibers are so great that one can seldom speak with assurance of a fatty degeneration in this tissue. These considerations lend strong support to the negative findings of Anderson and Rosenau.

The Arthus⁴ phenomenon seems to lend experimental support to the view that foreign protein may produce local anatomic changes when introduced into a sensitive animal.

Longcope⁵ studied the effects of repeated injections of foreign protein in guinea-pigs, rabbits, dogs and cats. Small sensitizing doses of horse serum or egg-white were given 20-25 days before the secondary injections were begun. The secondary injections were spaced and regulated with the object of producing shock a number of times. Some of the experiments lasted as long as 3 months, during which time as many as 15 injections were given. Not all injections resulted in anaphylactic symptoms and many of the reactions were recorded as slight.

Changes were found in the kidneys in 23 of 29 rabbits, and in a large proportion of the other animals experimented on. In the animals that died acutely diffuse degenerative changes were found. In those that lived longer, extensive round cell infiltration was the most striking change. Dense fibrous rays with atrophic tubules were found in 6 rabbits. Six rabbits showed normal kidneys at necropsy. Portal cirrhosis of the liver and areas of chronic myocarditis were also found frequently.

In a more recent paper Longcope⁶ found that similar though less marked renal lesions may be produced by a single large injection of foreign protein, or by several injections in which a different protein was used each time. All the sensitized animals developed nephritis,

⁴ Arch. internat. de Physiol., 1908-9, 7, p. 471.

⁵ Jour. Exper. Med., 1913, 18, p. 678.

⁶ Ibid., 1915, 22, p. 793.

but of those that received a single large injection, or several different proteins, only one-third developed nephritis. He concluded that foreign protein is itself toxic and may produce nephritis, but that previous sensitization increases the extent, severity and constancy of the lesions.

The lesions described by Longcope correspond in all respects with those of ordinary spontaneous nephritis. As controls, 24 supposedly normal rabbits were studied, and in 4 of these a few small areas of round cell infiltration were found similar to those occurring in the experimental animals. The argument that the lesions were produced by the foreign protein because they were more extensive and severe than in the controls has no value, since advanced stages of spontaneous nephritis are of common occurrence. The controls apparently did not have the same opportunity to contract an infection, since there is no mention of their having been kept under the same conditions and for the same length of time as the experimental animals. It is also to be noted that some of the animals may have had nephritis at the outset of the experiment, since it is admitted that animals with a faint trace of albumin were used. Again, there was no control at all for the healed stages of nephritis, of which 6 cases are mentioned and attributed to the action of the protein, since these do not cause albuminuria. No cultures were taken from the kidneys to determine whether the nephritis was due to infective or to chemical irritants.

Three of Longcope's experiments were controlled somewhat better. Three dogs were operated on, and a portion of one kidney from each was removed for examination before the secondary inoculations were begun. The excised portions of the kidneys were found normal, but each animal developed nephritis after a number of injections of protein subsequent to the operation. But no cultures were made from these kidneys, and a renal infection was therefore not excluded.

The uncertainty of the results of Longcope's work is indicated further by his attributing the ordinary cirrhosis of the liver of rabbits to the action of the protein. This is an unusually common lesion. It occurs in fully three-fourths of adult rabbits and is probably due to bacterial infection. All who have studied rabbit livers extensively recognize this as a spontaneous lesion.

Longcope's conclusions were confirmed by Boughton⁷ to the extent that repeated injections of foreign protein may produce renal lesions.

⁷ *Jour. Immunology*, 1916, 1, p. 105.

Boughton produced repeated anaphylactic shock in guinea-pigs, using beef serum and egg-white as antigens. Some of the animals received as many as 19 secondary injections, and the average number of injections was 9. The average duration of the experiments was 4 months, but some animals were under experimentation as long as 8 months. The urine was not examined systematically. Twenty-three guinea-pigs were used. Degeneration, desquamation, and necrosis of the tubular epithelium was the most striking change noted and was seen in all cases. Round cell infiltration was found in nearly all cases, though it was never very marked. There was no distinct scar formation.

Six controls were given injections of protein so spaced as to avoid the production of anaphylaxis. Tubular injury was much less marked than in the sensitized animals. Areas of round cell infiltration were also seen, but these were only one-tenth to one-fifteenth as frequent as in the sensitized animals.

All injections were given intraperitoneally and ranged as high as 10 c c (undiluted serum, or 50% solution of egg-white). The kidney lesions were regarded as subacute rather than chronic.

Boughton seems to conclude that foreign protein produces some renal injury when injected without the production of anaphylactic shock, and rather severe injury when this phenomenon is repeatedly produced. The tubular injury was the most prominent lesion, and the production of a typical chronic nephritis is apparently not claimed. As regards the interpretation of the round cell infiltration, the sources of error are the same as in Longcope's experiments.

EXPERIMENTS

The purpose of our investigation was to determine whether chronic nephritis can be experimentally produced in rabbits by a foreign protein. Repeated shock was produced in a few animals, but in most of the experiments no attempt was made to produce anaphylaxis.

Special effort has been made to eliminate various sources of error. All rabbits that had albuminuria were discarded. In order to eliminate the occasional inactive cases of spontaneous nephritis, rabbits with normal urine were operated on with aseptic precautions under ether anesthesia in order to expose and examine the surface of the left kidney. All except early cases of nephritis may be recognized by the

presence of spots and pits on the surface of the kidney. Both kidneys are nearly always involved to about the same extent, so that it is not necessary to examine the right kidney. About one week after the operation, the experiment was begun in case the wound was completely healed and the urine free from albumin. Ascitic fluid, egg-albumin, and sheep serum were used. Injections were made intravenously except as otherwise indicated.

ASCITIC FLUID WITH ANAPHYLAXIS

Rabbit 25.—Weight 2,000 gm. July 3: Test for albumin negative. Left kidney exposed and found normal.

July 12, 17, 24: 1 c c sensitizing injections of ascitic fluid.

Sept. 10: 1 c c, moderate anaphylactic symptoms.

Sept. 21: 0.5 c c, slight symptoms.

Oct. 5: 0.25 c c, slight symptoms.

Oct. 15: 0.5 c c, slight symptoms.

Oct. 25: 0.5 c c, moderate symptoms.

Nov. 5: 0.5 c c, moderate symptoms.

Nov. 15: 1 c c, severe symptoms.

Nov. 21: Found dead.

Tests for albumin negative, Sept. 10, 25, Oct. 11. Trace of albumin, Nov. 7. Seven secondary injections were given over a period of 71 days. At necropsy the kidneys showed no gross changes. Microscopically an occasional small focus of lymphocytes was found deep in the cortex.

Rabbit 35.—Weight 1,885 gm. Sept. 11: Test for albumin negative. Left kidney exposed and found normal.

Oct. 5: 0.25 c c, ascitic fluid.

Oct. 15: 0.5 c c, slight symptoms.

Oct. 25: 0.5 c c, moderate symptoms.

Nov. 5: 1 c c, moderate symptoms.

Nov. 15: 1 c c, slight symptoms.

Nov. 26: 2 c c, slight symptoms.

Dec. 6: 2.5 c c, moderate symptoms.

Dec. 17: 2.5 c c, slight symptoms.

Dec. 27: 2.5 c c, slight symptoms.

Jan. 10: Killed.

Tests for albumin negative, Nov. 8; positive, Jan. 4 and 10. On Dec. 5, the serum from this rabbit gave a strong precipitin test with human blood serum. Eight secondary injections were given over a period of 87 days. At necropsy the kidneys showed no gross changes. Microscopically an occasional small lymphocytic focus was found in the cortex.

Rabbit 33.—Weight 2,320 gm. July 18: Test for albumin negative. Left kidney exposed and found normal.

Sept. 10: 0.5 c c, ascitic fluid.

Sept. 21: 0.5 c c, severe symptoms.

Oct. 5: 0.25 c c, slight symptoms.

Oct. 15: 0.25 c c, slight symptoms.

Oct. 25: 0.5 c c, moderate symptoms.

Nov. 5: 0.5 c c, moderate symptoms.

Nov. 15: 1 c c, slight symptoms.

Nov. 26: 2 c c, slight symptoms.

Dec. 6: 2.5 c c, slight symptoms.

Dec. 17: Found dead.

Tests for albumin negative, Sept. 10, 25, Oct. 11, Nov. 7. On Dec. 4 the serum from this rabbit gave no precipitate with human blood serum. Eight secondary injections were given over a period of 87 days. The kidneys showed no lesions grossly or microscopically.

ASCITIC FLUID WITHOUT ANAPHYLAXIS

Rabbit 32.—Weight 2,320 gm. Oct. 2: Test for albumin negative.

Oct. 6: Left kidney exposed and found normal.

One c c ascitic fluid, Oct. 16, 23.

Two c c, Oct. 27, Nov. 1, 6, 12, 17.

Three c c, Nov. 22.

3.5 c c, Nov. 27, Dec. 3, 8, 13, 18, 22, 26, 31.

Tests for albumin negative, Oct. 13, Nov. 9, 22, Dec. 13; positive, Jan. 3.

Killed, Jan. 3. 16 injections were given over a period of 79 days.

No gross changes found in the kidneys. Microscopically a few small lymphocytic foci found in the cortex.

Rabbit 54.—Weight 2,105 gm. Jan. 28: Test for albumin negative.

Jan. 31: Left kidney exposed and found normal. Injections of ascitic fluid as follows: Feb. 11, 0.5 c c; Feb. 19, 0.75 c c; Feb. 23, 1 c c; Feb. 28, 0.75 c c; Mar. 5, 0.75 c c; Mar. 12, 1 c c; Mar. 19, 1 c c; Mar. 26, 1 c c; Apr. 3, 1 c c.

Killed, Apr. 8.

Albumin tests negative Feb. 14, 21, Mar. 2, 26, Apr. 5. Nine injections were given over a period of 56 days. No gross or microscopic lesions found in the kidneys.

Rabbit 65.—Weight 2,170 gm. Dec. 6: Albumin test negative. Left kidney exposed and found normal. Injections of ascitic fluid as follows: Dec. 22, 0.25 c c; Dec. 26, 0.5 c c; Dec. 31, 0.5 c c; Jan. 11, 0.75 c c; Jan. 22, 1 c c; Feb. 5, 1.5 c c (slight shock); Feb. 11, 1.5 c c; Feb. 19, 1.5 c c; Feb. 23, 2 c c; Feb. 28, 1 c c; Mar. 5, 1 c c; Mar. 12, 1 c c.

Killed Mar. 18. Twelve injections were given over a period of 86 days. Albumin tests negative, Dec. 14, Jan. 3, 30, Mar. 2. No gross or microscopic lesions found in the kidneys.

EXPERIMENTS WITH EGG-ALBUMIN

Rabbit 44.—Weight 2,250 gm. Jan. 3: Test for albumin negative.

Jan. 5: Left kidney exposed and found normal.

Injections of a 1% solution of egg-albumin as follows: Jan. 15, 0.25 c c; Jan. 22, 0.5 c c; Jan. 26, 0.75 c c; Jan. 30, 1 c c; Feb. 5, 1.5 c c; Feb. 11, 2 c c of a 50% solution of egg-white intraperitoneally. Death, Feb. 11.

Albumin tests negative, Jan. 15, 25, 30. At necropsy the kidneys were found slightly swollen and moist. There was precipitated albumin in the capsular spaces and in the lumina of the convoluted tubules. Whether this albumin was excreted egg-albumin or serum-albumin was not determined. There were no areas of lymphocytic infiltration.

Rabbit 50.—Weight 1,850 gm. Feb. 28: Albumin test negative. Left kidney exposed and found normal. Intraperitoneal injections of a 50% solution of

egg-albumin as follows: Mar. 14, 0.5 cc; Mar. 19, 1 cc; Mar. 26, 1 cc; Apr. 3, 1 cc; Apr. 8, 1 cc; Apr. 11, 1 cc; Apr. 16, 1 cc. Death, Apr. 19.

Albumin tests negative, Mar. 19, Apr. 5, 19. Seven injections were given during a period of 36 days. No gross or microscopic lesions found in the kidneys.

Rabbit 60.—Weight 1,560 gm. Dec. 5: Test for albumin negative.

Dec. 6: Left kidney exposed and found normal.

One cc of a 1% solution of egg-albumin was injected intravenously on the following dates: Dec. 20, 26, 31, Jan. 11, 22, and Feb. 5. One cc of a 50% solution of egg-white was injected intraperitoneally on Feb. 11, 19, 23, 25, and Mar. 5. Death, Mar. 9.

Albumin tests negative Dec. 14, Jan. 5, 15, 30, and Feb. 25. Trace of albumin, Jan. 25.

No gross changes found in the kidneys. Microscopically an occasional cast was observed, but there were no areas of lymphocytic infiltration.

EXPERIMENTS WITH SHEEP SERUM

Rabbit 38.—Weight 1,950 gm. Dec. 3: Albumin test negative. Dec. 6: Left kidney exposed and found normal. Injections of sheep serum, diluted with equal volume of salt solution, as follows: Jan. 19, 0.5 cc; Jan. 24, 0.75 cc; Jan. 29, 1 cc; Feb. 5, 1.5 cc. Found dead, Feb. 6.

Albumin tests negative, Jan. 28, Feb. 1. The kidneys were swollen and cloudy. Precipitated albumin was found in the lumens of some of the convoluted tubules. There were no areas of lymphocytic infiltration.

Rabbit 42.—Weight 1,750 gm. Jan. 4: Test for albumin negative. Jan. 15: Left kidney exposed and found normal. Jan. 19, 0.5 cc of sheep serum. Jan. 22, found dead. Kidneys were cloudy. No microscopic examination.

Rabbit 53.—Weight 2,970 gm. Feb. 7: Left kidney exposed and found normal. Feb. 14: Test for albumin negative. Injections of sheep serum as follows: Feb. 19, 0.5 cc; Feb. 23, 0.75 cc; Feb. 28, 0.5 cc; Mar. 5, 0.5 cc; Mar. 12, 0.5 cc; Mar. 21, 1 cc; Mar. 26, 1 cc; Apr. 3, 1 cc. Apr. 4, found dead.

Albumin tests negative, Mar. 12, 21. Kidneys showed no gross changes. Microscopically precipitated albumin was found in the lumina of many of the tubules. There were a few casts. No areas of lymphocytic infiltration seen.

Rabbit 63.—Weight 1,860 gm. Feb. 7: Test for albumin negative. Left kidney exposed and found normal. Injections of sheep serum as follows: Feb. 19, 0.5 cc; Feb. 23, 0.75 cc; Feb. 28, 0.5 cc; Mar. 5, 0.5 cc; Mar. 12, 0.5 cc; Mar. 21, 1 cc; Mar. 26, 1 cc; Apr. 3, 1 cc; Apr. 4, found dead.

Albumin tests negative, Feb. 14, Mar. 21. Trace of albumin, Mar. 12. Several small pits were found on the surfaces of each kidney. Microscopically there were small areas of lymphocytic infiltration corresponding to the surface pits.

Rabbit 67.—Weight 1,960 gm. Jan. 4: Test for albumin negative. Jan. 5: Left kidney exposed and found normal. Injections of sheep serum as follows: Jan. 19, 0.5 cc; Jan. 24, 0.75 cc; Feb. 5, 1 cc; Feb. 19, 1 cc; Feb. 23, 1.5 cc; Feb. 28, 1 cc; Mar. 5, 1 cc; Mar. 12, 1 cc; Mar. 21, 1 cc; Mar. 26, 1 cc; Apr. 3, 1 cc; Apr. 11, 1 cc; Apr. 16, 1 cc; Apr. 18, 0.5 cc. Killed, Apr. 30.

Albumin tests negative, Jan. 17, 28, Feb. 25, Mar. 14; positive, Apr. 4, 18. A number of fairly deep pits were seen on the surfaces of the kidneys. Microscopically rather extensive lymphocytic infiltrations were found underlying the

pits. The lesions correspond with those of a well advanced spontaneous nephritis.

Rabbit 113.—Weight 1,824 gm. May 14: Albumin test negative. Left kidney removed and found normal grossly and microscopically. Injections of sheep serum as follows: May 28, 0.5 cc; June 4, 0.75 cc; June 13, 2 cc; June 27, 2 cc; June 29, 1 cc; July 2, 2 cc. Killed, July 11. Loss of weight during experiment, 474 gm. Albumin tests positive, June 27, July 2, 10. The right kidney was covered with pits. Rather large areas of lymphocytic infiltration were found deep in the cortex underlying the pits, the appearances being those of a spontaneous nephritis. Cultures from the kidney in dextrose broth gave a pure growth of a streptococcus.

Rabbit 103.—Weight 1,250 gm. May 2: No albumin. Left kidney exposed and found normal. Injections of sheep serum as follows: May 28, 0.5 cc; June 4, 0.75 cc; June 6, 0.75 cc; June 13, 2 cc; June 27, 2 cc; June 29, 1 cc; July 2, 2 cc. Killed, July 6. No loss of weight. Albumin tests positive, June 27, July 3. The kidneys showed many fairly deep pits. Microscopically there were rather large areas of lymphocytic infiltration. The appearances were those of a spontaneous nephritis. Cultures from the kidneys taken immediately after death gave a pure growth of a streptococcus.

DISCUSSION

These experiments indicate that ascitic fluid does not injure the kidney of a rabbit when injected intravenously in moderate amounts. The occasional small foci of lymphocytes found at necropsy in the injected animals can hardly be attributed to the protein, since such lesions are commonly found in nonexperimental animals and were not excluded by the preliminary examination. Lesions of this size produce no changes on the kidney surface and seldom cause albuminuria.

The production of anaphylactic symptoms repeatedly did not cause nephritis. The symptoms were usually rather mild, but it is not possible to produce severe shock repeatedly. The animals either die or become refractory. The symptoms were apparently nearly as pronounced as in the experiments of Longcope and Boughton.

Egg-albumin in large doses seemed to cause a mild parenchymal injury of the kidney occasionally, but no chronic changes of any kind were produced. This protein seems to be only mildly toxic for rabbits.

Sheep serum, however, is evidently toxic for rabbits, especially in relatively large doses. Several animals died shortly after a single injection. It produces a tubular injury to the kidney, but this is not severe enough to cause the death of the animal. There is no evidence of special glomerular injury. Degenerative changes only were observed in the first three experiments; but during the summer inflammatory renal lesions were found in 4 rabbits. This is evidently the same

lesion which Longcope obtained. It undoubtedly developed during the course of the experiments, since it was not present at the beginning. It is similar in all respects to ordinary spontaneous nephritis. Like the spontaneous disease it would seem to be due to a bacterial infection, since pure cultures of streptococci were obtained from the kidneys of rabbits 113 and 103.

Our interpretation of these apparently positive results with sheep serum is, therefore, that the animals acquired an infection during the course of the experiments. This infection may have been contracted from other rabbits in the animal house, or it may have been introduced by the intravenous injections, as these were not made with complete aseptic precautions. In either case it is probable that the injury of the kidney by the sheep serum favored the development of the infection. The injured kidney may have been a *locus minoris resistentiae*.

If this interpretation be correct it may be said that injections of sheep serum cause nephritis in rabbits by injuring the kidney and giving opportunity for the lodgment and growth of bacteria which may gain access to the circulation; but this is not a nephritis due directly to the action of anaphylatoxin or foreign protein. It is conceivable that toxic foreign protein might have a similar effect in man, but it is unlikely that such proteins ever gain access to the circulation in such relatively large amounts as are used experimentally in animals. And finally, if we grant the possibility that toxic foreign protein may enter the circulation and injure the kidney, we would expect the development of lesions similar to those found in the rabbit. But the only human renal lesions comparable to spontaneous nephritis of the rabbit are the hematogenous infections, namely, abscess of the kidney, descending pyelonephritis, and acute interstitial nephritis. Aside from the arteriosclerotic kidney, nearly all cases of chronic nephritis in man are examples of chronic glomerulo nephritis which has no anatomic resemblance whatever to spontaneous nephritis in the rabbit. It is therefore clear that the experimental production of such a lesion as spontaneous nephritis does not throw any light on the etiology of chronic nephritis in man.

It is not improbable that the various experimenters, who believe they have produced a chronic nephritis experimentally with uranium, potassium chromate, foreign protein, streptococci, etc., have been dealing with a spontaneous nephritis which was contracted during the experiment and hastened in its development by renal irritants. But

even if all these claims were true we still have made little progress in determining the etiology of chronic nephritis in man.

As regards the etiology of human chronic nephritis we share the view, now widely held, that streptococci are the main causative agents, and that they first produce acute glomerular lesions which in many instances heal, but in others pass into a chronic stage. The continuous escape of streptococci from a focal infection or through a diseased mucous membrane would theoretically favor the development of chronic glomerular lesions. But thus far no one has been able to furnish experimental proof to support this theory.

SUMMARY

Nontoxic foreign proteins, such as ascitic fluid, do not produce renal injury of any kind in the rabbit when injected intravenously in moderate amounts. The repeated production of anaphylactic symptoms with this protein likewise does not injure the kidney.

Egg-albumin may produce slight parenchymal injury when injected in large amounts, but usually it causes no damage.

Toxic foreign proteins such as sheep serum produce considerable parenchymal injury to the kidney which in some instances seems to lead to the rapid development of a renal infection comparable in all anatomic respects to the ordinary type of spontaneous nephritis. The fact that streptococci were isolated in pure culture from the kidneys of such animals shows that the lesion is essentially infective, and not the direct result of foreign protein or anaphylatoxin.

No lesion comparable to human chronic glomerulo-nephritis has been produced experimentally.

There is no experimental evidence that foreign protein is in any way responsible for chronic nephritis in man.

SPONTANEOUS NEPHRITIS IN RABBITS AND ITS RELATION TO CHRONIC NEPHRITIS IN MAN

WITH ONE PLATE

E. T. BELL AND T. B. HARTZELL

From the Department of Pathology, University of Minnesota, and the National Dental Research Association

It is well known that spontaneous¹ nephritis is often found in rabbits, and there are frequent estimates in the literature as to its frequency. There is, however, not much detail published as to the finer structure and pathogenesis of this lesion, and it is still a serious source of error for those engaged in the study of experimental nephritis. It is noteworthy that no investigator who claims to have produced a chronic nephritis experimentally has been able to draw a sharp distinction between the spontaneous lesions and those supposedly produced by the experimental procedure. This fact is usually admitted, though sometimes ignored, and the argument is made that a lesion occurring much oftener in the experimental than in the control animals is due to the experimental procedure. It is not generally recognized that nephritis in animals (especially the rabbit) is entirely different in its pathogenesis from human chronic nephritis, and that therefore the successful experimental production of lesions of this character does not necessarily illuminate the problems of the etiology of human chronic nephritis. We have, therefore, thought it worth while to give a brief account of the etiology and pathogenesis of spontaneous nephritis and to call attention to its relations to the problems of human nephritis and experimental nephritis.

FREQUENCY

Smith² found some renal lesion in 11 of 50 rabbits. Ophüls,³ in a study of the kidneys of 50 rabbits, found 28 entirely normal, 9 with slight parenchymatous lesions, 3 with a few small areas of cellular infiltration, and 10 with "scattered small areas in which were marked interstitial lesions with formation of small depressions on the surface."

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¹ "Naturally acquired," suggested by MacNider, is a more accurate term than "spontaneous," but the latter seems to be in general use.

² *Arch. Int. Med.*, 1911, 8, p. 468.

³ *Proc. Soc. Exper. Biol. and Med.*, 1911, 8, p. 75.

Ghoreyeb⁴ mentions a spontaneous lesion in 9 rabbits in an article in which 48 rabbits are recorded by number. Longcope⁵ found lesions in the kidneys of 8 of 24 supposedly normal rabbits. Major⁶ found that of "52 rabbits dead of natural causes, none had the extensive scarring of the cortex" such as was found in his experimental animals. Apparently Major was writing only of severe lesions.

In our own experience, which includes the study of over 200 rabbits over a period of two years, the percentage with spontaneous nephritis varied greatly from month to month. Sometimes nearly all were found normal, but usually from 10-20% were diseased. There were two periods during the past two years, each lasting about 1 month, when over 80% of the rabbits examined had nephritis. The disease apparently assumes an endemic form at times. The animals used were purchased in large groups from different dealers, and it was noted that the disease was much more prevalent in some groups than in others when they were first put into the animal house. Many rabbits developed nephritis after they came into our possession.

In view of the great variations we have noted, it seems unsafe to conclude, as Longcope and others have done, that a given lesion is due to the experimental procedure merely because it occurs oftener in the experimental than in the control animals. The source of the animals used as controls and the length of time they are kept in the animal house are very important factors.

ETIOLOGY

We have attempted to isolate bacteria in eight cases of spontaneous nephritis by removing pieces of the kidney aseptically immediately after the animal had been killed, or from the living animal under ether anesthesia. The pieces of tissue were put in plain broth or in dextrose broth. Signs of growth first appeared in the medium after several days' incubation. Four of the cases gave no growth. In two cases we obtained a gram-positive staphylococcus in pure culture. These organisms when first isolated were very virulent — $\frac{1}{4}$ c c of a broth culture being a fatal dose for a rabbit. The third subculture, however, was practically avirulent.

In two other cases pure cultures of streptococci were obtained. These grew feebly and did not cause death or infection when inocu-

⁴ Jour. Exper. Med., 1913, 18, p. 29.

⁵ Ibid., 1913, 18 p. 678.

⁶ Jour. Med. Research, 1917, 35, p. 349.

lated into rabbits. We did not succeed in reproducing nephritis by injecting normal rabbits with the organisms obtained from any of the diseased kidneys. In the absence of this evidence the organisms isolated cannot with certainty be regarded as the etiologic agents; but it seems to us a highly probable assumption. They can hardly be due to contamination since the growth did not appear until after several days' incubation, the organisms were in pure culture, and in two instances were very virulent. It is known to us that streptococci have often been obtained from the tissues of rabbits, but these have usually been from animals that died slowly from natural or experimental disease where the opportunity for agonal invasion was present, and not from animals killed and cultured directly. Dr. W. P. Larson of this laboratory tells us that he has repeatedly found the normal kidneys of rabbits sterile.

PATHOLOGICAL ANATOMY AND GENESIS

For the sake of clearness we shall discuss this topic without special reference to observations recorded in the literature. In the earliest stages small foci of lymphocytes are found usually near the corticomedullary junction but sometimes elsewhere in the cortex. There are only a few of these lymphocytic foci in mild cases — many sections of the kidney may be entirely normal. But in severe cases these areas are numerous, and they may fuse to form extensive areas of infiltration. The lymphocytes are of intermediate size and lie for the most part closely packed between the tubules, but a few invade the epithelial cells (Fig. 1). At this early stage there are no gross changes on the surfaces of the kidneys. In severe cases the cut surface is clouded. Albumin is present in the urine except in cases where the foci are few and small.

The pressure of the exudate soon causes atrophy and disintegration of the parts of the tubules involved in the lymphocytic foci, and the destruction of these tubules is responsible for the changes that occur subsequently. The first gross changes noted are the appearance of a reddish or purplish spot on the surface of the kidney overlying the lymphocytic focus. Later on this purplish spot gradually sinks and a distinct funnel-shaped depression develops in its place. In the final stages the pit may be as deep as the thickness of the cortex. When the surfaces are studded with such deep pits the kidney assumes a shrunken scarred appearance (Fig. 2).

Microscopic sections through the purplish surface spot before the pit has developed show an early stage of atrophy of the cortical tubules belonging to the tubules destroyed in the lymphocytic foci. The atrophic tubules are smaller and more darkly stained than the normal, and their lumens are small or completely obliterated (Fig. 3). As the atrophy progresses these tubules become gradually converted into narrow epithelial cords, and in the final stages even these have disappeared entirely. Sections through pits such as are shown in Fig. 2 show complete atrophy of the tubules, but the shrunken glomeruli are easily identified (Fig. 5).

The tubules atrophy from the lymphocytic focus to the tip of the medullary pyramid as well as toward the surface of the kidney. This is easily seen by the study of serial sections of such a kidney as is shown in Fig. 2. The scar is readily traced from the surface pit down through the medulla to the tip of the pyramid. In the medulla it stands out very prominently as a band of tissue from which the tubules have disappeared (Fig. 4). The end-result of the lymphocytic infiltration at the cortico-medullary junction is the formation of a wedge-shaped scar with its apex at the tip of the pyramid and its base at the surface of the kidney. The lymphocytes usually occur only near the center of the wedge. That this is the shape of the lesion can be determined in any case by the study of serial sections.

We are not dealing therefore with a diffuse interstitial nephritis such as occurs in diphtheria and scarlet fever, but with discrete lymphocytic foci which cause atrophy of the entire tubule system by destroying its central portions. In severe cases where the foci fuse the appearance of acute interstitial nephritis is simulated.

The gross appearance of the kidneys in the late stages depends on the number of lymphocytic foci originally present. There may be only 2 or 3 pits, or the pits may be so closely set that the kidney has a shrunken contracted appearance. These severe extensive cases have given rise to the view that spontaneous nephritis in the rabbit is comparable to the contracted kidney of man, but this is not a correct interpretation. The most common form of chronic nephritis in man is a glomerulo-nephritis in which the primary injury is to the glomerulus, and the atrophy of the tubule is subsequent to the destruction of this structure. In the human arteriosclerotic kidney areas of tissue, of small or large size, undergo atrophy because of sclerosis of the nutrient artery. In spontaneous nephritis in the rabbit neither

of these conditions obtains, but the atrophy results from destruction of a part of the tubule by an inflammatory exudate. The only form of human nephritis in any way comparable to rabbit nephritis is a form of focal infection in the kidney characterized by abscesses or diffuse inflammatory areas, and due to hematogenous infection. It is often referred to as "excretory" nephritis, or excretory pyelonephritis when the infection passes on into the pelvis of the ureter.

It is clear, therefore, that the successful solution of the problem of spontaneous nephritis in the rabbit will not necessarily throw any light on the problems of human nephritis. As far as we have observed spontaneous nephritis in animals other than the rabbit is similar to rabbit nephritis, but we have not studied other animals extensively enough to warrant a general statement. Le Count⁷ described a case of chronic glomerulo-nephritis in a rabbit, and MacNider⁸ reported this form as common in dogs. The important problem for experimental pathology in this connection is the production of a chronic glomerulo-nephritis.

Spontaneous nephritis frequently results in recovery. This is especially true in cases where the infection is not very extensive. Albumen disappears from the urine and the functional test is normal, but the pits on the surface persist. There is no attempt at regeneration of the scar tissue. In many cases, however, there is a progressive destruction of renal tissue. Death usually results from toxemia and intercurrent diseases rather than from renal insufficiency.

URINARY FINDINGS

Albuminuria is usually demonstrable in the active stages of the disease. It may be intermittently present in mild cases, and it disappears entirely after healing. This last point is of considerable importance in experimental work. We have found deeply pitted and scarred kidneys at operation in several animals which had normal urine and normal phthalein excretion just before the operation. The presence of albumin in the urine does not necessarily mean that the rabbit has nephritis of the type described above. Our records show seven animals with albuminuria in which necropsy revealed only slight tubular injury in the kidney, the cause of which could not be clearly established. It is well known that albuminuria may be produced by a large variety of renal irritants. Casts are not constantly present.

⁷ *Jour. Infec. Dis.*, 1914, 15, p. 389.

⁸ *Jour. Med. Research*, 1916, 34, p. 177.

RENAL FUNCTION

The phenolsulphonephthalein test shows no change except in severe cases. The lowest 2-hour excretion we have recorded is 20%. When the phthalein test was reduced to any considerable degree extensive renal involvement was always present. Healed cases gave a high excretion, even though the scarring was considerable.

DEVELOPMENT OF NEPHRITIS IN CONTROL ANIMALS

We have records of two rabbits with normal urine in which exploratory operation showed the surfaces of the kidneys smooth. These animals were kept as controls for some other experiments. About three months after the exploratory operation they were found to have developed a typical spontaneous nephritis. This observation is of great importance in interpreting experimental results. It is easily possible for nephritis to develop during the course of long experiments independently of the experimental procedure.

Another type of lesion occasionally seen has been described by several authors (Le Count,⁷ Fig. 11, p. 402). It consists of a wedge-shaped area composed of dilated tubules with flattened epithelium. We have not worked out the genesis of this lesion.

ASSOCIATED LESIONS

In all cases of spontaneous nephritis there was an associated lymphocytic infiltration of the portal connective tissue of the liver. This chronic hepatitis is also present in many cases in which the kidneys are normal. About 75% of the livers examined by us (50 cases) showed portal infiltration in varying degrees. Organisms may usually be cultivated from these livers but they do not always correspond with those obtained from the kidneys.

We did not make an extensive routine examination of the heart muscles in our nephritic cases; but in three rabbits in which large numbers of sections of heart muscles were examined we found foci of lymphocytes similar to those described by Longcope⁹ and attributed by him to the action of foreign protein. It is probable that this form of myocardial infection is frequently associated with spontaneous nephritis.

⁹ Jour. Exper. Med., 1915, 22, p. 793.

RELATION OF SPONTANEOUS TO EXPERIMENTAL NEPHRITIS

There has been no confusion of spontaneous with experimental acute nephritis since the two lesions are entirely different; but the natural disease has seldom if ever been sharply differentiated from chronic lesions supposedly produced by experimentation. As indicated in preceding paragraphs no observer has offered any convincing evidence that the lesion supposedly produced differs anatomically from the spontaneous disease. In fact, the illustrations accompanying many articles correspond entirely with the appearances of the natural disease. The usual argument is that a lesion occurring much oftener in experimental than in control animals must be due to the experimental procedure (O'Hare;¹⁰ Dickson;¹¹ Christian, Smith and Walker;² Longcope¹²). But other explanations of these results are possible. 1. A spontaneous nephritis may develop during the course of experiments lasting several months. The control animals were not kept in the laboratory for the same length of time under the same conditions. Klotz¹³ admits this as a possible source of error in his work. 2. The use of renal irritants such as uranium nitrate may favor the development of an infection in the kidneys, because of the general lowered resistance caused by the drug or because injured renal tissue is more susceptible than normal. The investigators who used uranium did not make cultures of the kidneys to see if bacteria were present. We obtained a growth of bacteria from one uranium kidney.

Observers who apparently did not examine the urine at the beginning of each experiment, and there are several in this group, are open to the criticism that the animals may have had nephritis at the outset of the experiment.

When virulent bacteria were injected into the blood stream, as in the experiments of Le Count,⁷ Klotz,¹³ and Hartzell and Henrici¹⁴ it is easily possible that they are entirely responsible for the resulting lesions. One would expect a lesion resembling spontaneous nephritis from this procedure. The acute glomerular lesions obtained with streptococci suggest a relationship between these organisms and human glomerulo-nephritis; but the other lesions which various experimenters have attributed to injected bacteria bear no anatomic resemblance to the human disease.

¹⁰ Arch. Int. Med., 1913, 12, p. 49.

¹¹ Ibid., 1909, 3, p. 375.

¹² Jour. Exper. Med., 1913, 18, p. 678.

¹³ Trans. Assn. Am. Phys., 1914, 29, p. 49.

¹⁴ Jour. Am. Med. Assn., 1915, 64, p. 1055.

SUMMARY

Spontaneous nephritis in the rabbit is a subacute or chronic infectious disease characterized in the early stages by lymphocytic infiltrations in the kidneys usually at or near the cortico-medullary junction.

The parts of the tubules included in each lymphocytic focus undergo atrophy, and subsequently there is atrophy of the tubule systems both proximally and distally to the lymphocytic foci. The end-result is the formation of wedge-shaped scars with their apices at the apex of the pyramid and their bases formed by pits on the surface.

In our experience nephritis is much more common in some groups of rabbits than in others. The sanitary environment seems to be important. The disease is especially apt to develop in animals kept in the laboratory for several months.

Albuminuria is present in the active stages of the disease, but is absent in healed cases. Unlike human chronic nephritis many cases recover.

The investigators who believe they have produced chronic nephritis experimentally have not had controls adequate to exclude the spontaneous disease, and the lesions described correspond closely with spontaneous lesions.

No chronic glomerulo-nephritis has been produced experimentally. There is no anatomic or pathogenetic relationship between spontaneous nephritis in the rabbit and human chronic glomerulonephritis.

EXPLANATION OF PLATE

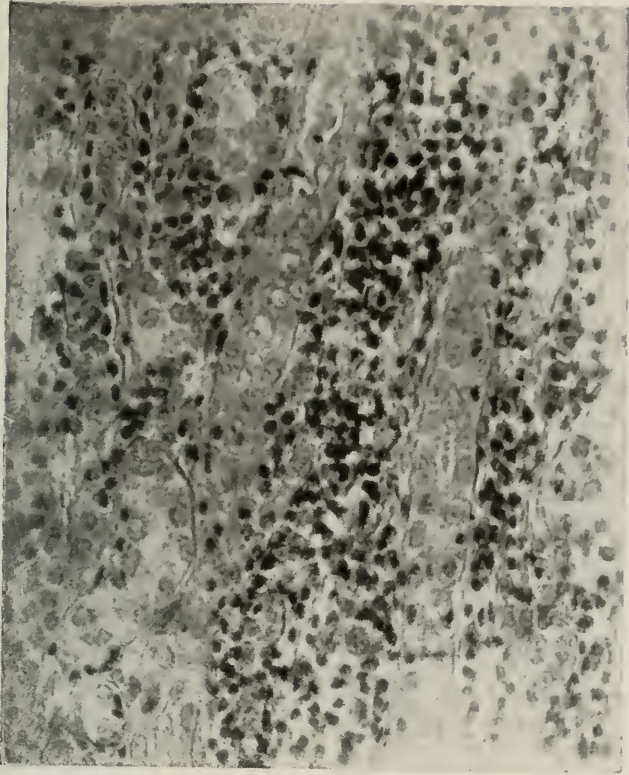
Fig. 1.—Photomicrograph. Area of lymphocytic infiltration at the cortico-medullary junction. Some tubules are already destroyed. This is the earliest type of lesion in spontaneous nephritis.

Fig. 2.—Photograph. Kidney showing numerous deep pits due to atrophy of cortical areas. The atrophic areas were produced by lymphocytic infiltrations at the cortico-medullary junction.

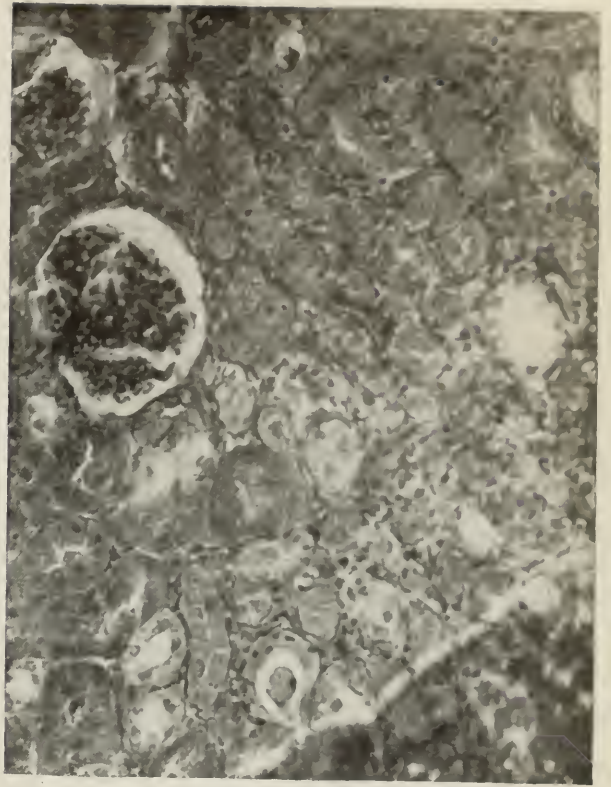
Fig. 3.—Early stage of atrophy of convoluted tubules due to destruction of their corresponding collecting tubules in a lymphocytic focus at the edge of the medulla. (The tubules in the lower left hand part of the figure are normal.)

Fig. 4.—Scar in medulla. This was traced in serial sections and found to connect with a surface pit such as is shown in Fig. 5.

Fig. 5.—Cortical pit (scar) showing complete disappearance of all the tubules. The glomeruli are still present. This is a healed lesion.



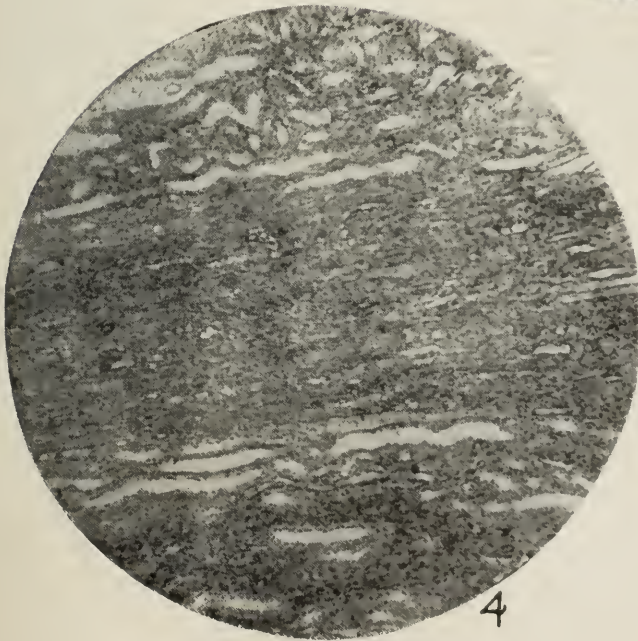
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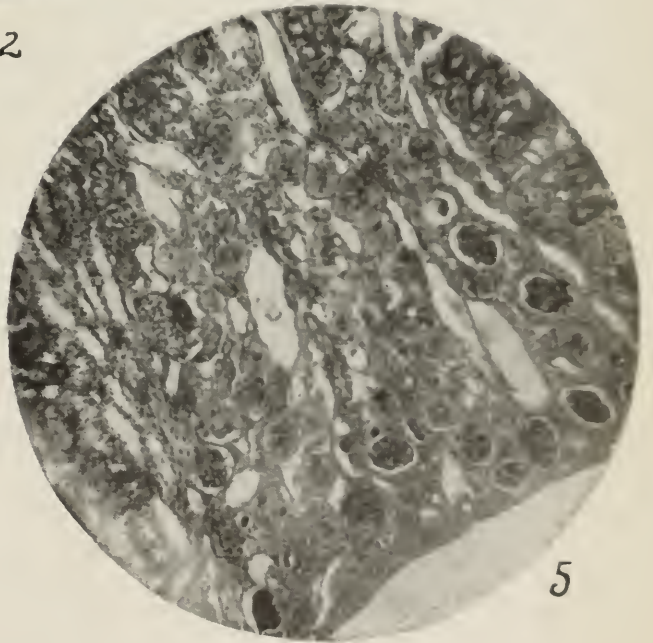
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THE PROTECTION AFFORDED BY VARIOUS FILTERS AGAINST BACTERIAL SUSPENSIONS IN AIR

RICHARD C. TOLMAN, ERNEST W. GUERNSLY,
VERNE D. CHARLESTON, AND ROBERT H. DOUGHERTY

The work described in this article was undertaken at the suggestion of Dr. John Johnston, secretary of the National Research Council, and Dr. Peyton Rous, vice-chairman of the Division of Medicine and Allied Sciences, with a view to selecting a filtering material which would afford adequate protection for physicians, nurses and others obliged to work in rooms, the air of which is infected with bacteria. Tests which have been previously made establish the degree of prevention given by various types of cloth masks against the projection of infectious droplets in coughing and talking, but we know of no study made on the penetration through mask materials of bacteria suspended in convection currents of air, which, whether dry or moist, are certainly not held in droplets of moisture.

METHOD OF STUDY

1. *The Bacterial Cultures.*—*Bacillus prodigiosus* was selected for these experiments because (1) the red pigment which it develops in plate cultures makes it possible to recognize accidental contamination in the plates; (2) its size, one of the chief factors influencing penetration of mask materials, is representative, and (3) it is nonpathogenic.

The cultures were prepared by inoculating sterile beef broth from a 20-day old culture which contained enough *prodigiosus* to impart a deep red color to the solution. One small platinum loopful of the 20-day broth culture was used for each 5 cc of sterile broth. The new culture was incubated for 18 hours at 37.5° C. The incubation was not continued longer because of the tendency to form clumps in the broth which might clog the atomizer in setting up the suspension. It later appeared that it would have been better to use more concentrated culture in smaller quantity and toward the end of the work a few determinations were made in which the old red 20-day culture was used.

2. *The Production of Bacterial Suspensions.*—The bacterial suspensions were set up in a 1 cu. m. sheet metal box which had been previously disinfected with formaldehyd and was filled with air forced through a copper spiral heated in a bath of molten Woods metal. The suspension was formed by spraying the pure broth culture of *prodigiosus* in the form of a very fine mist through a hole in one corner of the box. The evaporation of the mist carrying the bacteria was facilitated by a layer of calcium chlorid spread over the floor.

A fan directing a gentle current of air upward under the incoming spray helped to keep the bacteria in the air until they could be freed of their load of water. A preliminary warming of the broth culture to 40° C. before spraying, helped make the evaporation of the water more rapid. Even with these refinements only a small percentage of the bacteria originally introduced were actually obtained in suspension, due to the falling out of the larger drops of the spray, the lodging of droplets and of bacteria on the side of the box, etc. The fact that all the droplets of mist sprayed into the box actually evaporated, was shown by the lack of any deposition of moisture in the glass tubes through which the samples of air to be tested were drawn from the box.

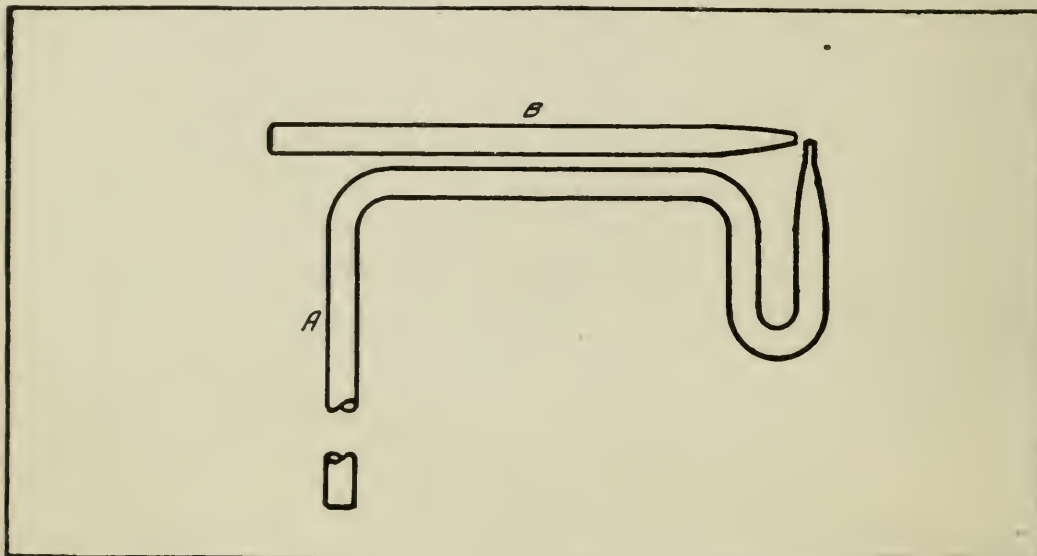


Fig. 1.—Showing the atomizer made from glass tubing used for spraying the culture into the box.

The atomizer used for spraying the culture into the box was made from glass tubing as shown in Fig. 1. The solution to be sprayed is drawn up through tube A and out of the capillary by the aspirating action of a fine jet of air which is forced through the capillary of tube B. The capillary openings of the tubes were extremely fine and the particular atomizer used was selected from a number because it gave the finest spray. Air was supplied to the atomizer at a rate of 15 liters per minute by means of a Type A Leiman Bros. pump driven by a $\frac{1}{4}$ H. P. motor. To prevent contamination the air for the aspirator was drawn from the box itself. The liquid was sprayed at a rate of approximately 10 cc per minute.

The usual amount of the 18-hour culture used was about 20 cc. When only 10 cc were used the concentrations of bacteria obtained was not as high as desired; and 40 cc gave only slightly higher concentrations of bacteria than did 20 cc. The character of the suspension was, of course, not greatly different when different amounts were used, since in any case the droplets which did not evaporate—whether because they were too large or because the box became saturated with moisture—fell out and there remained in the air only the bacteria, more or less moist.

3. *The Method of Carrying Out the Test.*—The general plan of the test was to draw a known volume of suspension through the filter, catching the bacteria

which penetrated the filter in a liquid medium; and simultaneously to draw through a parallel path, not containing a filter, the same volume of suspension, catching the bacteria in this path in the same way. A comparison of the number of bacteria found in the two paths then gives a value for the percentage penetration of the filtering material. The simple apparatus used is shown in Fig. 2.

The filter is held in a standard $1\frac{1}{2}$ " pipe union in which the inside flange has been ground away smooth. The filter rests in the fitting in the same way a bushing would be held except that the filter extends across the opening. Four-inch nipples extend from each side of the union, and are fitted with rubber stoppers to receive the glass tubing which forms the rest of the path.

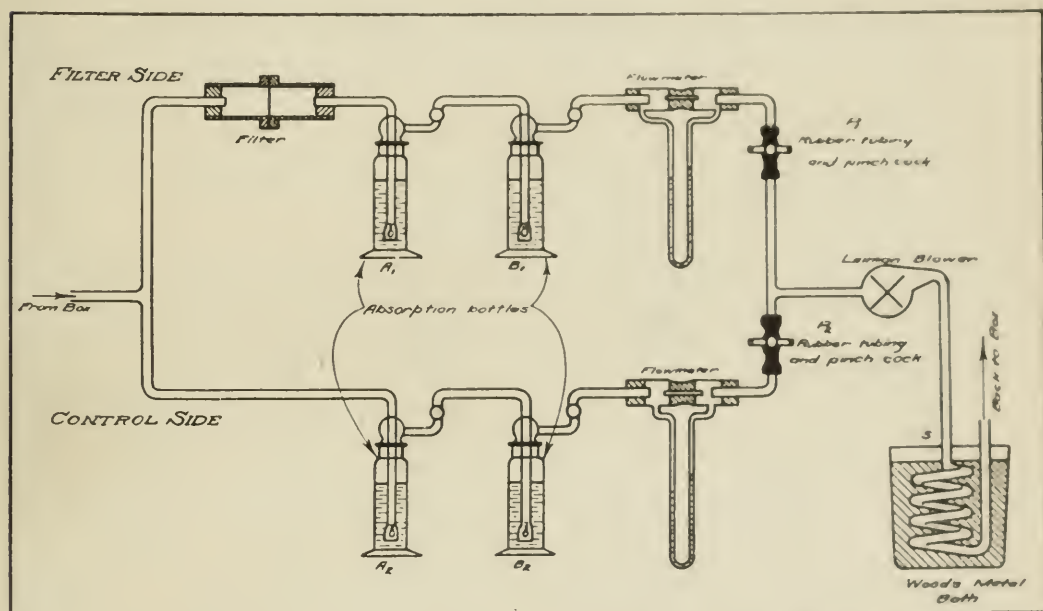


Fig. 2.—Diagram of the apparatus employed in these experiments.

The absorption bottles, A_1 , B_1 , A_2 and B_2 are standard gas absorption bottles of 300 cc capacity and about 20 cm. in height. Special apron bubblers are provided on the gas inlet tubes. Flowmeters in each path after the absorption bottles measure the flow which is kept at the proper value by means of pinch cocks P_1 and P_2 . The air is pulled through the two parallel paths by means of the same Leiman blower that is used in spraying the broth culture. In order that the partial vacuum created by the drawing out of the sample will not cause contaminated air to be drawn into the box through leaks, the air drawn out is passed through the heated copper spiral (S) and back into the box. The air after passing the spiral goes through a long tube and before reaching the box cools to about 50° C. as was shown by a thermometer.

Before each determination, all of the sampling apparatus between the box and the flowmeters was sterilized in steam at 20 pounds pressure for half an hour. The filter was afterward dried at a low temperature in the oven.

The original plan was to use beef broth as the medium for catching the bacteria. It was found, however, that beef broth frothed so badly in the absorption bottles as to make it impractical for this purpose. Physiologic salt solution was found to be satisfactory and was used in all of the determinations.

included in this report. The amount used in each of the bottles was about 200 c c or somewhat less.

The rate of flow of air through the filter used in these experiments was 6 liters per minute and the area of filtering material exposed about 12 sq. cm. This rate corresponds fairly closely with the normal breathing rate over the effective area in ordinary cloth face masks and is much higher than breathing rates with areas, which can easily be obtained with masks of proper design; so that results obtained at this rate cannot be too favorable to the mask under test.

In the earlier determinations, one 10-minute sample was taken in each run, starting about one-half minute after the spray was stopped, which gave time for any remaining mist particles to settle out or evaporate. Later, however, as the technic became simplified, two samples were tested in each run, in order to see if the bacteria would become more penetrating with further chance to dry. The first sample was from 0-5 minutes after stopping the spray, and the second from 20-25 minutes. In some of the runs the fan inside the box was allowed to run throughout the test, while in others it was stopped after completing the spray and in three experiments was allowed to run while the first sample was being taken, and then stopped.

4. *Plating Out the Bacteria for Counting.*—As soon as possible after getting the bacteria into the salt solution in the manner outlined, aliquot portions of the solution were mixed with warm sterile beef broth containing 2% of agar-agar and poured into Petri dishes of 10 cm. diameter which had been sterilized for 1 hour in dry air at 150° C. When it was desired to obtain a higher dilution than could be obtained by pipetting directly from the bottles, a small portion was diluted with sterile salt solution and an aliquot portion of this plated out. By repeating this dilution any proportion of the total number of bacteria which was suitable could be obtained in the Petri dish. In the earlier determinations before the approximate concentration of bacteria to be expected was known, as many as 5 different dilutions ranging from 1:100 to 1:1,000,000 were used for each of the four bottles. Later, however, 2 dilutions for each bottle were found sufficient.

The colonies developing from the bacteria in the plates were counted after 24 hours' incubation at 37.5° C., since at this time the colonies were sufficiently developed to be seen fairly well and molds and other accidental contaminations were less confusing than later. The count was always checked at 48 hours. Calculations were based on the plates giving a count nearest 100; since, if the colonies were too thick, there might be a suppression of colonies, while if they were too few the error in assuming the plate to be a fair sample would be great.

In the early tests there were frequent contaminations in small amounts, apparently of *Staph. aureus*, *Staph. albus*, *proteus* and some molds which latter, however, developed so late as to cause no confusion. Later, with improved technic, contaminations became even less troublesome, there being many plates without any stray organisms at all.

The plating out of the bacteria was rarely delayed longer than 20 minutes after completing a sample. Two duplicate determinations were made which showed that *B. prodigiosus* was not killed in this time in the salt solution, plate cultures taken over an interval of an hour after collection of a sample failing to show any appreciable falling off in the concentration of bacteria.

FILTERS STUDIED

The following filters were examined to determine the protection which they afforded:

1. Buttercloth masks as recommended by Doust and Lyon.¹
2. Buttercloth masks as above except moistened as in wearing.
3. Buttercloth masks as above except wet.
4. A buffed felt of medium hard texture about $\frac{1}{8}$ " thick, made by the Booth Felt Co., and giving a pressure drop of about 35 mm. of water with a flow of 500 cc of air per sq. cm. per minute.
5. A special buffed felt of hard texture about $\frac{5}{32}$ " thick, made by the Felters Co., of a blend of 20 California, 40 Cape and 40 Texas, and giving a pressure drop of about 130 mm. of water with a flow of 500 cc of air per sq. cm. per minute.

TABLE 1
PENETRATION OF PRODIGIOSUS THROUGH MEDIUM HARD FELT
Sample 0-10 minutes

	Dilution	No. Colonies	No. Bacteria in Bottle
Control Bottle A	1:1,400	514	719,600
	1:14,000	120	1,680,000
	1:140,000	17	2,380,000
Control Bottle B	1:1,800	20	36,000
	1:18,000	3	54,000
Filter Bottle A	1:1,800	8	14,400
	1:18,000	1	18,000
Filter Bottle B	1:1,300	0	0
	1:13,000	0	0

No. of bacteria in 60 liters of unfiltered air..... 1,716,000
 No. of bacteria in 60 liters of filtered air..... 14,400
 Percentage penteration of filter..... 0.8%

20 c c 18-hour culture was used to set up suspension. The fan in the box ran throughout the experiment.

6. An electrical precipitation tube. This apparatus is part of an "Electric Mask" developed by Dr. Arthur B. Lamb and associates. It operates on the same principle as the familiar Cottrell precipitators used to remove dust and fumes from the fuel gasses of blast furnaces and cement kilns. The apparently hopeless difficulty of securing adequate and dependable high tension electric power in a portable equipment has been solved by the development of a very light and efficient storage battery and induction coil. This equipment as at present developed will operate continuously from 6-10 hours and weighs only about 5 pounds.

EXPERIMENTAL RESULTS

The accompanying tables illustrate the tests. Filter bottle A and filter bottle B are the first and second absorption bottles after the

¹ Jour. Am. Med. Assn., 1918, 71, p. 1216.

TABLE 2
PENETRATION OF PRODIGIOSUS THROUGH THREE LAYERS OF BUTTERCLOTH
Sample 0-5 minutes

	Dilution	No. Colonies	No. Bacteria in Bottle
Control Bottle A	1:1,600	2,000	3,200,000
	1:16,000	250	4,000,000
Control Bottle B	1:1,740	800	1,392,000
	1:17,400	93	1,618,200
Filter Bottle A	1:1,720	1,300	2,236,000
	1:17,200	143	2,459,600
Filter Bottle B	1:165	221	36,465
	1:1,650	21	34,651

Sample 20-25 minutes

Control Bottle A	1:164	50	8,200
	1:1,640	4	6,560
Filter Bottle A	1:176	42	7,392
	1:1,760	4	7,040

Sample 0-5 minutes:
 No. bacteria in 30 liters unfiltered air..... 5,618,200
 No. bacteria in 30 liters filtered air..... 2,494,251
 Percentage penetration through filter... 44%
 Sample 20-25 minutes:
 No. bacteria in 30 liters unfiltered air..... 8,200
 No. bacteria in 30 liters filtered air..... 7,392
 Percentage penetration through filter..... 90%
 Remarks: 20 c c 36-hour culture sprayed into box. Fan stopped after spraying.

TABLE 3
PENETRATION OF PRODIGIOSUS THROUGH THREE LAYERS OF WET BUTTERCLOTH
Sample 0-5 minutes

	Dilution	No. Colonies	No. Bacteria in Bottle
Control Bottle A	1:1,320	69	91,080
	1:13,200	7	92,400
Control Bottle B	1:105	260	27,300
	1:1,050	19	19,950
Filter Bottle A	1:123	50	6,150
	1:1,230	10	12,300

Sample 20-25 minutes

Control Bottle A	1:156	195	30,420
	1:1,560	22	34,320
Filter Bottle A	1:182	7	1,274
	1:1,820	1	1,820

Sample 0-5 minutes:
 No. bacteria in 30 liters unfiltered air..... 111,030
 No. bacteria in 30 liters filtered air..... 6,150
 Percentage penetration through filter..... 5.5%
 Sample 20-25 minutes:
 No. bacteria in 30 liters unfiltered air..... 34,320
 No. bacteria in 30 liters filtered air..... 1,274
 Percentage penetration through filter..... 3.7%
 Remarks: The filter was wet by spraying water onto it just before the experiment. Examination just after determination showed it to be thoroughly saturated with water. 10 c c of 18-hour culture was sprayed to produce the suspension. The fan ran throughout the experiment.

TABLE 4
PENETRATION OF PRODIGIOSUS THROUGH THE ELECTRICAL PRECIPITATION TUBE
Sample 0-5 minutes

	Dilution	No. Colonies	No. Bacteria in Bottle
Control Bottle A	1:1,550	3,000	4,650,000
	1:15,500	400	6,200,000
Control Bottle B	1:176	5,000	880,000
	1:1,760	550	968,000
Filter Bottle A	1:120	0	0
	1:1,200	0	0
Filter Bottle B	1:170	0	0
	1:1,700	0	0

Sample 20-25 minutes

Control Bottle A	1:160	1,800	288,000
	1:1,600	207	331,200
Filter Bottle A	1:160	0	0
	1:1,600	0	0

Sample 0-5 minutes:

No. bacteria in 30 liters unfiltered air..... 7,168,000
 No. bacteria in 30 liters filtered air..... 0
 Percentage penetration through filter..... 0%

Sample 20-25 minutes:

No. bacteria in 30 liters unfiltered air..... 331,200
 No. bacteria in 30 liters filtered air..... 0
 Percentage penetration through filter..... 0%

Remarks: 5 c.c of old red culture was sprayed for the suspension. The fan ran throughout the experiment.

TABLE 5
PENETRATION OF PRODIGIOSUS THROUGH HARD FELT
Sample 0-5 minutes

	Dilution	No. Colonies	No. Bacteria in Bottle
Control Bottle A	1:2,080	3,000	6,240,000
	1:20,800	353	7,342,400
Control Bottle B	1:2,020	361	729,220
	1:20,200	43	868,600
Filter Bottle A	1:202	0	0
	1:202	0	0

Sample 20-25 minutes

Control Bottle A	1:1,840	103	189,520
	1:18,400	8	147,200
Filter Bottle A	1:200	0	0
	1:200	0	0

Sample 0-5 minutes:

No. Bacteria in 30 liters unfiltered air..... 8,211,000
 No. bacteria in 30 liters filtered air..... 0
 Percentage penetration through filter..... 0%

Sample 20-25 minutes:

No. bacteria in 30 liters unfiltered air..... 189,520
 No. bacteria in 30 liters filtered air..... 0
 Percentage penetration through filter..... 0%

Remarks: 10 c.c old red culture was sprayed to produce suspensions. Fan ran for first five minutes only.

filter, and control bottles A and B are the absorption bottles in the parallel path in which there is no filter. From the number of colonies actually counted and the dilution, both of which are given in the table, the number of bacteria present in each bottle is calculated. Since bottles A and B are in series on both the filter and the control side, the results for the bottles A and B in both sets are additive. As explained earlier, the counts on which the penetration calculation is based are those nearest 100 colonies per plate.

Table 6 gives in summary the results on the various filters studied.

TABLE 6
THE PENETRATION OF *BACILLUS PRODIGIOSUS* THROUGH FILTERS

Test	Filter	First Sample 0-5 Minutes		Second Sample 20-25 Minutes			Remarks
		Bacteria per Liter Original Air	Per- centage Bacteria Passing Filter	Bacteria per Liter Original Air	Per- centage Original Concen- tration	Per- centage Bacteria Passing Filter	
V	3 layers buttercloth	73,250	73	185	0.25	85	20 c c 18-hour cul- ture. Fan stopped after spraying
VI	3 layers buttercloth	38,430	50	272	0.71	76	20 c c 18-hour cul- ture. Fan ran throughout
VII (Table 2)	3 layers buttercloth	187,273	44	273	0.15	90	20 c c 36-hour cul- ture. Fan stopped after spraying
XIII	3 layers buttercloth moistened as in wearing	31,320	28.5	3,103	10.0	21	20 c c 18-hour cul- ture. Fan stopped 5 minutes after spraying
VIII (Table 3)	3 layers buttercloth saturated with water	3,701	5.5	1,144	30.0	3.7	10 c c 18-hour cul- ture. Fan ran throughout
I (Table 1)	Medium felt	Sample 0-10 min. 28,600 0.8		20 c c 18-hour cul- ture. Fan ran throughout
II	Medium felt	222,173	0.35	481	0.21	8.5	20 c c 72-hour cul- ture. Fan stopped after spraying
III	Medium felt	9,541	0.61	553	5.8	2	40 c c 18-hour cul- ture. Fan stopped after spraying
IV	Medium felt	232,750	4.5	70,433	30.0	2.5	5 c c old red cul- ture. Fan ran throughout
IX	Hard felt	2,562	0	133	5.2	0	10 c c 18-hour cul- ture. Fan ran throughout
XII	Hard felt	5,404	0	6	0.1	0	20 c c 18-hour cul- ture. Fan stopped 5 minutes after spraying
XIV (Table 5)	Hard felt	273,700	0	6,317	2.3	0	5 c c old red cul- ture. Fan stopped 5 minutes after spraying
X (Table 4)	Electrical precipita- tion tube	228,933	0	11,040	4.6	0	5 c c old red cul- ture. Fan ran throughout
XI	Electrical precipita- tion tube	131,213	0	17,520	13.0	0	5 c c old red cul- ture. Fan ran throughout

1, 2 and 3. *Buttercloth Masks Recommended by Doust and Lyon.*—These masks consist of three layers of buttercloth, a closely woven gauze. They were shown by Doust and Lyon to prevent completely the projection of infectious material by a person wearing the mask. Our investigation shows, however, that they are by no means adequate protection to the wearer against bacteria which having become freed from their envelop of water, are swept up in the air by convection currents. A penetration of from 44 to 73% was observed for the more moist 0-5 minute sample, while for the 20-25 minute drier sample, 76% penetration was the lowest value observed. In these tests, care was taken that the fabric should be held loosely as in wearing and not stretched. Our results show that moistening the buttercloth increases the filtering efficiency of these masks. A degree of moisture similar to that which might in extreme cases obtain in wearing, lowers the penetration of the buttercloth to 29%. One determination was made with the buttercloth saturated with water in order to exaggerate this effect and in this case a penetration of only 5.6% was found.

4. *Medium Hard Felt.*—This felt affords almost perfect protection against bacterial suspensions. From 2 to 4% penetration is the maximum under the most favorable conditions.

5. *Hard Felt.*—This felt affords complete protection against suspensions of *B. prodigiosus* under every condition.

6. *Electrical Precipitation Tube.*—In two duplicate determinations, made in the same way as the determinations on the cloth filters, the portable electrical precipitation mask developed by Dr. Arthur B. Lamb was found to prevent completely the passage of live bacteria. To find out whether the bacteria were killed in the action of the precipitating tube, the tube was rinsed out after a test with 100 c c sterile salt solution and portions plated out with beef broth and agar-agar in four different dilutions. All four plates remained entirely blank showing that the bacteria are killed by the electrical precipitation tube.

THE BACTERIAL CONCENTRATIONS ACHIEVED AND THE PERSISTENCE OF BACTERIAL SUSPENSIONS

It perhaps will be noticed that there are wide variations in the bacterial concentrations attained under apparently parallel conditions. Thus the concentration given by 20 c c of 18-hour culture sprayed into the box, varies from 5,404 bacteria per liter to 73,250 bacteria

per liter. This variation was due to various factors such as variation in the concentration of the mother culture used in inoculating, differences in the temperature of the box, differences in the efficiency of the spray due to the partial clogging by clumps in some instances, and perhaps other factors.

In the early tests portions of the culture to be sprayed were plated out in several dilutions in order to get an idea of the maximum possible bacterial concentration and the efficiency with which the bacteria were gotten into suspension. Even at a dilution of $1:10^{14}$, the colonies in the plate were much too thick to count. Estimating the number of colonies at 1,000,000 the maximum concentration obtainable in the box would be 10^{17} bacteria per liter as compared with 73,233, the maximum concentration actually obtained.

There was also considerable irregular variation in the persistence of the suspension even when the fan in the box was handled in the same way. The percentage ratio of the concentration at 20-25 minutes to that at 0-5 minutes is listed in Table 6 under the head of "Percentage of Original Concentration." The values for the "Percentage of Original Concentration" are shown below:

Fan Stopped Just after Spraying		Fan Stopped 5 Min. after Spraying		Fan Ran Throughout	
Test	Per Cent.	Test	Per Cent.	Test	Per Cent.
V	0.25	XIII	10.0	VI	0.71
VII	0.15	XII	0.1	VIII	30.0
II	0.21	XIV	2.3	IV	30.0
III	5.8			IX	5.2
				X	4.6
				XI	13.0

From these figures it is evident that the fan helps to support the suspension.

The decreased number of live bacteria found in the air at 20 minutes could, of course, be accounted for by the death of the bacteria as well as by their falling out. However, since in some instances the percentage of original concentration is as high as 30% at 20 minutes it seems likely that the decrease could be accounted for solely by the falling out of the bacteria in suspension. It is believed that the bacteria out in the open air would remain in suspension longer than in a box such as was used, since in the box they are constantly being blown against the top and sides to which they adhere.

Comparison of Penetrating Power of 1st and 2nd Sample.—In general, a higher percentage penetration was obtained for the second or drier sample than for the first. It is certain that the sample taken after 20 minutes was as dry, and therefore as penetrating, as the bacteria of similar size to be found in ordinary air.

Mask Recommended.—On the basis of the information presented in this article we have prepared masks with filters made from the hard felt. The filters have an area of approximately 125 sq. in., thus giving a much lower rate of air flow in actual breathing than that used in the bacterial tests. The pressure drop for the whole mask at ordinary breathing rates is only about 1 inch of water. These masks are available for test by suitable hospital authorities if desired.

SUMMARY AND CONCLUSION

Tests have been made to determine the degree of protection offered by various filtering materials against suspensions in air of *B. prodigiosus*.

Buttercloth masks have been found to give inadequate protection against bacteria in this form.

Felts are available which give complete protection against air-borne bacteria of the size of *B. prodigiosus*.

The portable electrical precipitation apparatus of Dr. Lamb gives complete protection against bacterial suspensions, killing the bacteria in its action.

A mask with a felt filter with an area of 125 sq. in. and a pressure drop of about 1 inch of water has been constructed which will give complete protection.

The efficiency of the buttercloth filter is greatly increased by wetting the cloth. It might be possible to arrange an efficient wet filter mask which would be much simpler, more comfortable, and cheaper than those in which felt is used.

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